NORTHWESTERN UNIVERSITY

Cellular and Molecular Mechanisms of Androgen Actions in Male Reproduction

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

For the degree

DOCTOR OF PHILOSOPHY

Field of NEUROBIOLOGY & PHYSIOLOGY

By

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EVANSTON, ILLINOIS

December 2008

ABSTRACT

Cellular and Molecular Mechanisms of Androgen Actions in Male Reproduction

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In male mammals, testosterone (T) is critical for sexual differentiation during development and reproductive success in adulthood. This thesis examines the cellular and molecular processes that mediate T's actions in the male brain and reproductive axis. Although T is clearly necessary for sexual behavior, the cellular mechanisms by which it enhances neuronal excitability are not well understood. I proposed that ATP-sensitive potassium (K^+_{ATP}) channels, which regulate cell excitability, play a role in male sexual behavior. The present studies demonstrate that T inhibits mRNA expression of the K^+_{ATP} channel subunit Kir6.2 in the male rat preoptic area and medial basal hypothalamus, and that pharmacological blockade of neural K^+_{ATP} channels restores copulation in castrates. These findings support the hypothesis that K^+_{ATP} channels serve as a mechanism by which T stimulates male sexual behavior.

Many of T's effects are exerted through conversion to estradiol (E_2). Estrogen receptor alpha (ERα) mediates E_2 actions in the male gonads and brain and is critical for normal sexual behavior and T biosynthesis. In the classical pathway, ERα binds to estrogen response elements (EREs) to regulate gene transcription. However, E_2 also exerts effects independently of EREs. This thesis assessed whether ERE-independent ERα signaling can rescue the disrupted masculine sexual behaviors and enhanced androgen biosynthesis that result from ERα gene deletion. I utilized male ERα null mice that possess an ERα knock-in mutation ("AA"), in which the mutant ERα cannot bind to DNA and only signals through ERE-independent pathways ($ERα^{-/AA}$ mice). I observed that male sexual behavior was not rescued in the $ERα^{-/AA}$ mouse. By contrast, ERE-

independent signaling restored serum T levels, testicular T secretion *in vitro*, and steroidogenic enzyme gene expression and activity. These data demonstrate that binding of ERα to EREs mediates E_2 stimulation of male sexual behavior, while ERE-independent ERα signaling mediates E_2 inhibition of T production.

Together these data contribute to our knowledge of the cellular and molecular mechanisms underlying T and E_2-ERα regulation of male reproductive function. Understanding steroid actions in specific target tissues and physiological systems will ultimately aid in the development of pharmacological therapies for male reproductive dysfunction.

List of Abbreviations

17β-HSD	17 beta hydroxysteroid dehydrogenase
2-DG	2-deoxy-D-glucose
3β-HSD	3 beta hydroxysteroid dehydrogenase
AA	Alanine substitutions of the ER knock-in mutation
aCSF	Artificial cerebral spinal fluid
ACTH	Adrenocorticotropin hormone
AF1, AF2	Activation function 1, activation function 2
ANOVA	Analysis of variance
AP-1	Activator protein 1
AR	Androgen receptor
ARE	Androgen response element
ArKO	Aromatase knockout mouse
ATD	1,4,6-androstatriene-3,17-dione
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C/EBPβ	CCAAT/enhancer binding protein beta
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAPON	C-terminal PDZ domain ligand of neuronal nitric oxide synthase
CBG	Corticosteroid binding globulin
cDNA	Complementary deoxyribonucleic acid

CF	Control female
CM	Control male
CNS	Central nervous system
CREB	cAMP response element binding protein
CREM	cAMP response element binding modulator
CRH	Corticotropin releasing hormone
CRH-BP	Corticotropin releasing hormone binding protein
CRH-R	Corticotropin releasing hormone receptor
CV	Coefficient of variance
Cx	Castrated
DA	Dopamine
DBD	DNA binding domain
DHT	Dihydrotestosterone
DMEM	Dulbecco modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPN	Diarylpropionitrile
E_2	17 beta estradiol
EGF	Epidermal growth factor
EPM	Elevated plus maze
ERα	Estrogen receptor alpha
ERαβKO	Estrogen receptor alpha beta double knockout

ERαKO	Estrogen receptor alpha knockout
ERβ	Estrogen receptor beta
ERβKO	Estrogen receptor beta knockout
ERE	Estrogen response element
EST	Expressed sequence tag
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GPR30	G-protein-coupled receptor 30
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
i.c.v.	Intracerebral ventricular
i.p.	Intraperitoneal
IGF-I	Insulin-like growth factor
IL-6	Interleukin - 6
Int	Intact
K^+	Potassium
K^+_{ATP}	ATP-sensitive potassium channel
Kir	Inwardly rectifying potassium channel
Kras2	Kirsten rat sarcoma viral oncogene homologue 2
LDL	Low-density lipoprotein
L-DOPA	L-3,4-dihydroxyphenylalanine

LH	Luteinizing hormone
MALS	Mammalian LIN-seven protein
Mapt	Microtubule-associated protein tau
MARTA1	MAP2 RNA trans-acting protein
MBH	Medial basal hypothalamus
$MgCl_2$	Magnesium chloride
MPOA	Medial preoptic area
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
$P450_{17\alpha}$	P450 17 alpha hydroxylase/$C_{17\text{-}20}$ lyase
P450scc	P450 side chain cleavage
PAK	p21-activated kinase
PCR	Polymerase chain reaction
PDZ	PSD-95/discs large/zona occludens-1
PLC-PIP2	Phospholipase C - Phosphatidylinositol-4,5-bisphosphate
POA	Preoptic area
PPT	1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole
PSD	Postsynaptic density
PVN	Periventricular nucleus
RIA	Radioimmunoassay

RNA	Ribonucleic acid
RPL19	Ribosomal protein L19
RT-PCR	Reverse transcription polymerase chain reaction
SDN	Sexually dimorphic nucleus
SEM	Standard error of the mean
SF-1	Steroidogenic factor 1
StAR	Steroidogenic acute regulatory protein
SUR	Sulfonylurea receptor
T	Testosterone
Tcf4	Transcription factor 4
TF	Testosterone-treated female
TH-ir	Tyrosine hydroxylase-immunoreactive
Tmod2	Tropomodulin 2
Tol	Tolbutamide
Veh	Vehicle
Veli	Vertebrate homologue of LIN-7
WT	Wild-type

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INTRODUCTION

In male mammals, testicular testosterone (T) and its metabolites are essential for successful reproduction. During critical periods of pre- and perinatal development, T secreted from the testis is responsible for the sexual differentiation of internal and external genitalia and is necessary to masculinize and defeminize the male brain and reproductive behaviors from the inherent feminization program (1, 2). These permanent, organizational effects are in contrast to the activational effects of T that occur in adulthood, such as the development and maintenance of secondary sex characteristics, stimulation of spermatogenesis, regulation of the hypothalamic-pituitary-gonadal (HPG) axis, and stimulation of sexual behavior. Although many of these organizational and activational effects are well characterized, the underlying mechanisms of steroid action are not fully understood.

Steroids stimulate sexual behavior in adulthood primarily through relatively slow effects on gene transcription, but sexual activity also requires rapid, interactive behavioral responses to relevant stimuli (3). Thus, steroids must affect neural responsiveness in order to quickly process these stimuli and translate them into an appropriate behavioral output. Accordingly, steroids alter the level of spontaneous electrical activity of anatomically defined populations of neurons within the brain and change the responses of central neuronal pathways (4-6). Increased neuronal excitability renders neurons more sensitive to the hormonal, chemical, and somatosensory inputs they receive, presumably increasing the likelihood of producing steroid-dependent behavioral responses. An objective of the present research was to explore a potential mechanism by which testosterone increases neuronal excitability in brain regions involved in sexual behavior. I propose that testosterone regulates ATP-sensitive potassium (K^+_{ATP}) channels based on evidence that these channels, (1) regulate cell excitability, (2) are expressed throughout brain regions involved in male sexual behavior, (3) are regulated by other steroid hormones, and (4) modulate

neurotransmitter release (7-14). These channels have not previously been studied as targets of T action and their role in the central control of male sexual behavior is not understood.

Although there is evidence for the requirement of the androgen receptor (AR) in the development and function of the male reproductive tract and masculine behaviors (15), T's effects are exerted largely through its conversion to estradiol (E_2) by the enzyme P450 aromatase and subsequent signaling through the estrogen receptor (ER), of which there are two forms, ERα and ERβ. The importance of E_2 action in male fertility is demonstrated by descriptions of testicular dysfunction and behavioral deficits in estrogen receptor alpha knockout (ERαKO) mice. Although prenatal development of the reproductive tract does not depend on ER, ERαKO males are infertile due to atrophy of the testes and seminiferous tubules, tubule dysmorphogenesis, reduced sperm counts, and impaired copulation and other sexually motivated behaviors (16-19). In contrast to ERα, deletion of ERβ does not impair testicular function, spermatogenesis, or normal masculine sexual behavior in adult mice (20-23). Thus, ERα plays a predominant role in the development and maintenance of normal male fertility.

E_2 typically elicits a response in target cells via activation of classical intracellular steroid receptors, which generally act as ligand-dependent transcription factors. In this classical pathway, ERα binds to estrogen response elements (EREs) to regulate gene transcription. However, emerging evidence demonstrates that the molecular mechanisms of ER signaling are more complex. ERα can also participate in several non-classical pathways, including ERE-independent gene transcription via protein-protein interactions with transcription factors, and rapid, membrane-initiated non-genomic pathways (24). To date, the relative roles of classical and non-classical ERα signaling pathways in estrogen regulation of male reproductive function and sexual behaviors have not been examined.

As testosterone is involved in so many aspects of male physiology and reproduction, normal regulation of its production is critical. In addition to tight control by the hypothalamic-pituitary-gonadal axis, it has been demonstrated that endogenous estrogens produced from within the Leydig cell, as well as exogenous estrogens, exert inhibitory actions on T synthesis and secretion. Recent evidence demonstrates that estrogens act via ERα, at least in part by a direct effect on androgenic capacity of individual Leydig cells (25). However, it is unclear whether classical and/or non-classical ERα signaling mechanisms mediate E_2's negative effects on androgen biosynthesis.

An objective of the present research was therefore to examine the contributions of classical and non-classical ERα signaling mechanisms to E_2 regulation of male reproductive physiology and behavior. Specifically, these studies assessed the degree to which ERE-independent ERα signaling can rescue the disrupted masculine sexual behaviors and enhanced androgen biosynthesis that have been shown to result from ERα gene deletion. I utilized ERα null mice that possess an ER knock-in mutation (E207A/G208A; "AA"), in which the mutant ERα cannot bind to DNA but retains activity in ERE-independent pathways ($ER\alpha^{-/AA}$ mice) (26). The $ER\alpha^{-/AA}$ model provides an exciting new opportunity to distinguish between ERE-dependent and ERE-independent mechanisms of E_2 action *in vivo*. Understanding the molecular mechanisms of ERα action will be helpful in developing pharmacological therapies that differentiate between ERE-dependent and –independent processes.

This dissertation examines the cellular and molecular mechanisms by which testosterone and estrogen exert their effects on male reproductive physiology and sexual behavior. The following chapter is a review of the current literature describing T and E_2's effects on sexual behavior and reproductive hormone secretions in the male. It also highlights evidence describing

several signaling mechanisms of ERα action. As much of our understanding of steroid hormone action has been obtained from studies of steroid hormone receptor gene knockout mouse models, data from ERαKO male mice are provided to serve as a background for the studies presented in subsequent chapters that utilize the non-classical ERα knock-in mouse model. Experiments in Chapter II examine K^+_{ATP} channels as a mechanism by which testosterone regulates neuronal activity and sexual behavior in the male rat. The effects of T on K^+_{ATP} channel subunit expression and the effects of a pharmacological K^+_{ATP} channel inhibitor on sexual behavior were analyzed. Chapters III and IV use the non-classical ERα knock-in mouse model ($ER\alpha^{-/AA}$) to examine the role of ERE-independent ERα signaling *in vivo*. In Chapter III, the sexual behavior of these males was evaluated to determine if the knock-in mutation could rescue the deficits in sexual behavior that are present in $ER\alpha^{-/-}$ male mice. In Chapter IV, hormone measurements in intact animals were evaluated to determine if non-classical ERα signaling mechanisms contribute to regulation of testosterone or gonadotropin levels in the male. The role of non-classical, ERE-independent ERα signaling in E_2 regulation of androgen biosynthesis was further examined by measuring *in vitro* T production and the expression and activities of steroidogenic enzymes. The role of classical and non-classical ERα signaling in E_2 regulation of stress responsiveness, anxiety, and depression is also examined in Appendix I. Appendix II presents data from a microarray analysis of the effects of prenatal androgen exposure on gene expression in the preoptic area of the hypothalamus. The results presented here provide insight into the mechanisms by which steroid hormones regulate normal male physiology and sexual behavior, and will potentially be useful in developing strategies to treat sexual or testicular dysfunction disorders.

CHAPTER I: LITERATURE REVIEW

A. Steroid Receptors in Male Reproduction

In addition to acting on target cells directly, T exerts its effects through conversion to metabolites estradiol (E_2) and 5α-dihydrotestosterone (DHT) (Figure 1). T and DHT, the two principal androgens in circulation, are both potent activators of the nuclear androgen receptor (AR). DHT is formed by the enzyme 5α-reductase in the testes and target tissues, and possesses approximately five-fold greater affinity for AR than T (27). E_2 is formed by the enzyme aromatase, and acts by binding to the estrogen receptor (ER), of which there are two forms, ERα and ERβ.

The enzyme 5α-reductase is expressed in liver, skin, epididymis and other accessory sex glands. Mutations in the 5α-reductase type 2 gene lead to 5α-reductase deficiency and cause male pseudohermaphroditism, in which virilization of the external genitalia does not occur. Aromatase is expressed in a number of tissues, including the testes, brain, liver, adipose tissue, and skin. Within the testes, aromatase is highly expressed in both Sertoli cells and Leydig cells, and is responsive to gonadotrophic-stimulated cAMP. In the brain, aromatase is expressed primarily in the preoptic area of the hypothalamus and the limbic system where it mediates T metabolism to E_2 during prenatal and neonatal development to induce sexual differentiation (28). Accordingly, blocking aromatization leads to feminization of neural structures and behaviors in adulthood. The levels of brain aromatase decline in the male brain in late fetal development but still contribute to E_2 synthesis in adulthood. Aromatase is regulated primarily by androgens, but only in some cells of the adult brain, namely those within the preoptic area, and male brains appear to have a greater capacity for androgen stimulation of aromatase activity than female brains (29). Aromatase deficiency is not lethal, but causes defects in reproductive function in men. Studies of aromatase knockout (ArKO) mice demonstrate that males display infertility at

older ages, arrest of spermatogenesis, Leydig cell hyperplasia, insulin resistance, and visceral adiposity. Therefore, E_2 production in the male is essential for normal sexual development, reproductive function, normal growth and metabolism.

1. Androgen Receptors

a. Structure and Function

The androgenic steroids T and DHT elicit biological responses in target cells via activation of the classical intracellular androgen receptor (AR), which typically acts as a ligand-inducible transcription factor (30-33). AR has greatest sequence similarity to the progesterone, mineralocorticoid, and glucocorticoid receptors, and contains structural motifs characteristic of the nuclear receptor superfamily (as described in detail for estrogen receptors in section A.2 below). The key functional domain of AR is the DNA-binding domain, which interacts with androgen response elements (AREs) in the promoter region of target genes.

b. Tissue Distribution in the Male

AR is present in a number of peripheral tissues in the male, including skeletal muscle, heart, kidney, liver, skin, and hair follicles. Within the male reproductive tract, AR is highly expressed in the testes, prostate, seminal vesicles and epididymis (34). AR has also been characterized biochemically in both cytosolic and nuclear extracts of brain tissue (35-37). In the adult rat brain, high levels of nuclear AR are present in the ventral medial nucleus of the hypothalamus and medial amygdala; intermediate levels are present in the arcuate nucleus-median eminence, medial preoptic nucleus, periventricular preoptic area, basal nucleus of the stria terminalis, anterior hypothalamus, periventricular anterior hypothalamus, lateral septum,

and parietal cortex; and low levels are seen in the lateral preoptic nucleus and cortical amygdala (35-42). Other reports have demonstrated the presence of AR in the hippocampus (43, 44).

Autoregulation of AR by androgens occurs at the level of both mRNA and protein and appears to be tissue-, species- and sex-specific (35, 45-50). For example, AR is generally down-regulated by androgens in neuronal tissue (44), while expression is predominantly up-regulated by androgens in non-neuronal tissue (51-53). In addition to its autoregulation, AR may be regulated by neurotransmitters and other hormones, including estrogen (37, 45, 54, 55).

c. Signaling Mechanisms

ARs appear to act predominantly as ligand-inducible transcription factors. In the classical pathway of androgen signaling, ligand-bound AR translocates to the nucleus and undergoes conformational changes that allow the DBD to interact with AREs in target gene promoters. The ligand-bound AR complex recruits tissue-specific co-regulatory proteins, which are required for chromatin modification and gene activation (56).

Androgens have also been observed to exhibit rapid, non-genomic effects in neuronal tissue (57) and within reproductive, musculoskeletal, cardiovascular and immune systems (58). There is evidence that the classical intracellular AR and/or novel, membrane-associated androgen binding proteins may mediate these membrane-initiated effects (58). Additional evidence suggests that AR possesses intrinsic hormone-independent transcriptional activity (59).

2. Estrogen Receptors

a. Structure and Function

ERα and ERβ are distinct proteins produced from two separate genes on the sixth and fourteenth chromosomes, respectively, and contain structural motifs characteristic of the nuclear receptor superfamily (Figure 1): a nonconserved N-terminal A/B transactivation domain; a highly conserved, central, zinc-finger-containing DNA-binding domain (DBD); and a high-affinity C-terminal E/F ligand-binding domain. An additional hinge region, positioned between the DNA-binding domain and the C-terminal region, contains the nuclear-targeting signal that causes ligand-bound receptors in the cytoplasm to translocate to the nucleus. The A/B domain contains a ligand-independent transactivation function AF1, while the E domain contains the ligand-dependent transactivation function AF2; the activities of AF1 and AF2 depend upon the target promoter and cell type (60). The DNA- and ligand-binding domains of ERα and ERβ share 97% and 60% sequence homology, respectively. Thus, these receptors interact with the same DNA response elements and have similar binding affinities for some endogenous, synthetic, and naturally occurring estrogens (61). However, ERα and ERβ also exhibit differences in their ligand-binding and transcriptional properties (62, 63).

b. Tissue Distribution in the Male

Specific responses of a target cell to estrogen depend, at least in part, on the amount and type of receptor present. ERα and ERβ have both overlapping and differential tissue distributions (64). In the adult mouse reproductive tract, ERβ has been reported to be present in Sertoli cells and Leydig cells but absent in spermatocytes (65), and ERα is highly expressed but limited to the Leydig cells and epididymis (64, 66, 67). Both ERα and ERβ have been identified

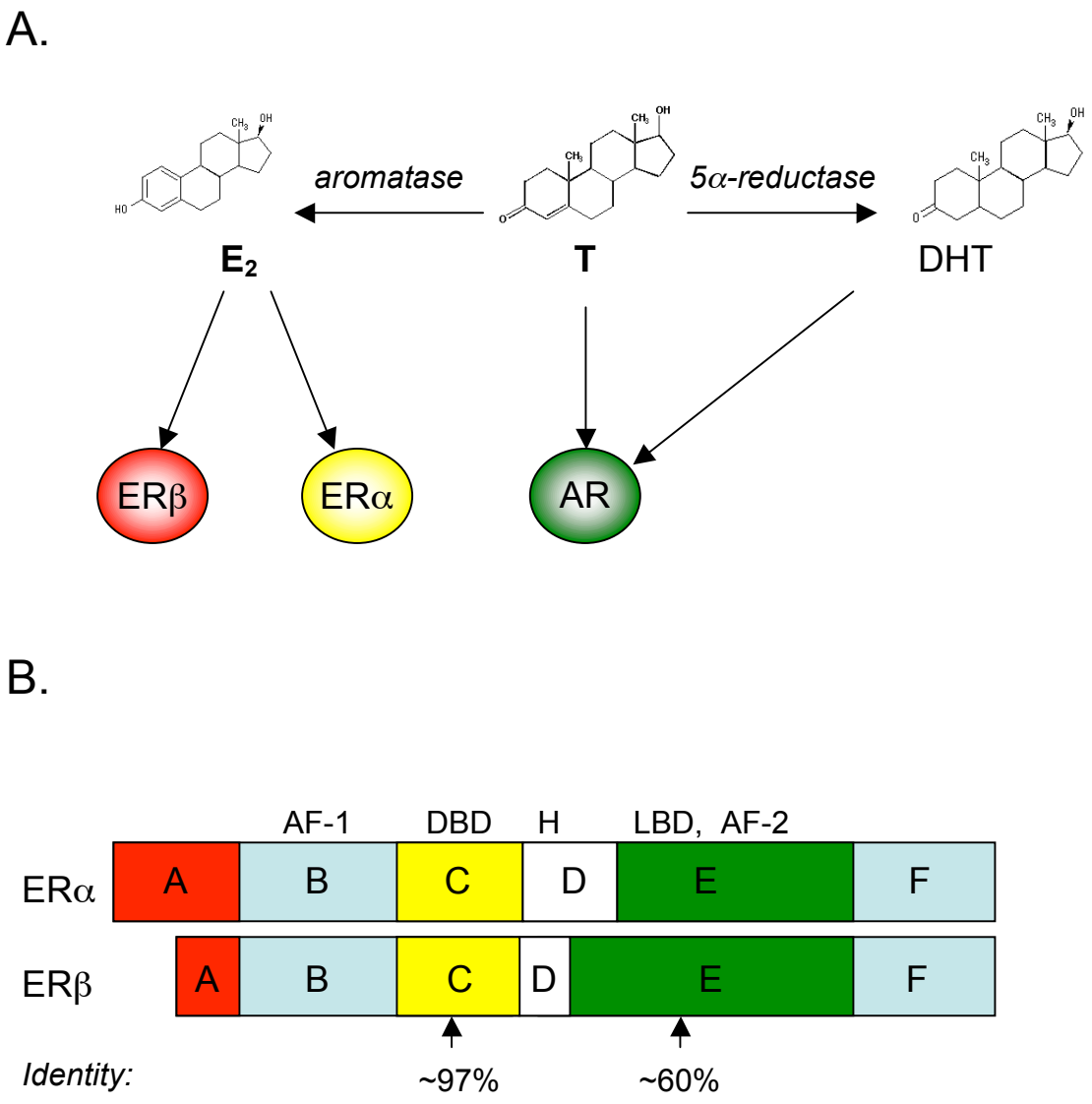


Figure 1. Steroids and steroid receptors. (A) Testosterone (T) and its 5α-reduced metabolite dihydrotestosterone (DHT) bind and activate androgen receptors (AR). T can also be aromatized to estradiol (E_2), which binds to the estrogen receptor, of which there are two forms, ERα and ERβ. (B) ERα and ERβ contain structural motifs characteristic of the nuclear receptor superfamily. The N-terminal consists of the A/B domain, the C domain forms the DNA-binding domain (DBD), and domains D/E/F constitute the ligand-binding domain (LBD). A hinge region (H) within the D domain contains the nuclear targeting signal. The AF-1 and AF-2 activation functions are localized to the DBD and LBD, respectively. ERα and ERβ are almost identical in the DBD but are different in the LBD, which accounts for their ligand specificity.

in numerous sites in the brain. For example, ERα and ERβ are both present in the hippocampus, medial nucleus of the amygdala, arcuate nucleus and periventricular preoptic nucleus of the hypothalamus, while ERα is present in the ventromedial nucleus and ERβ in the paraventricular nucleus, cerebellum and cortical regions (61, 68-70).

ERα and ERβ function as both homodimers and heterodimers. Some studies have reported that ERα tends to form homodimers, while ERβ prefers to heterodimerize with ERα, but others have reported that both receptors preferentially form heterodimers (63, 71-74). Regardless, heterodimer transcriptional activity appears to be equal to or slightly less than that of ERα homodimers (63, 73, 74). Additional evidence demonstrates that ERβ attenuates the ligand-activated transcriptional activity of ERα (75). Thus, ERα:ERβ ratio may determine overall estrogen responsiveness in tissues that express both subtypes.

c. Signaling Mechanisms

i. Genomic, ERE-dependent ER Signaling

In the classical pathway of estrogen action, upon binding of ligand, the estrogen receptor dissociates from heat shock proteins, dimerizes, and undergoes conformational changes that allow the DNA-binding domain to interact with estrogen response elements (EREs) present in the promoter region of target genes (76). The ligand-bound complex additionally recruits tissue-specific co-activator and co-repressor proteins, which are required for chromatin modification and gene activation or repression (77). Although ERs have traditionally been thought of as nuclear, ligand-dependent transcription factors, the molecular mechanisms of estrogen action are more complex. Only about one third of estrogen-responsive genes in humans contain EREs or ERE-like sequences (78). It is now recognized that E_2 actions are mediated by at least three other

"non-classical" ER pathways: (1) genomic, ERE-independent signaling, (2) rapid, non-genomic signaling, and (3) ligand-independent ER signaling (Figure 2).

ii. Genomic, ERE-independent ER Signaling

ERs can regulate gene expression independently of direct DNA binding at EREs. Instead, ERs may modulate the function of other transcription factors via protein-protein interaction, sometimes referred to as "transcriptional cross talk" (79). For example, ER has been shown to interact with Fos and Jun proteins (80), which bind to activator protein 1 (AP-1) sites, and mediate estradiol activation of genes such as ovalbumin (81), IGF-I (82), collagenase (83), and cyclin D1 (84, 85). ER interaction with AP-1 complexes also mediates estradiol repression of genes such as choline acetyltransferase (86). Similar tethering between ER and the transcription factor Sp1 mediates estradiol activation of low-density lipoprotein (LDL) receptor (87), *c-fos* (88), and cyclin D1 (89) genes. Furthermore, ER interaction with nuclear factor κB (NF-κB) or CCAAT/enhancer binding protein β (C/EBPβ) mediates repression of the IL-6 gene by estradiol (90, 91). Importantly, the actions of ERs at non-ERE binding sites often depend on the ligand, cell type, and receptor subtype (92, 93).

Additionally, estrogen-responsive genes that lack EREs may bind ERs at ERE-half sites, and ERα (but not ERβ) is able to bind directly to sites for the orphan nuclear hormone receptor SF-1 (78, 94). Thus, while the majority of ERE-independent genomic actions do not require direct binding to DNA, there is some evidence of ERE-independent ER signaling that still involves an interaction between the receptor and DNA. In fact, even when ER-DNA binding isn't required, specific residues within the zinc finger structure of the DBD may still be

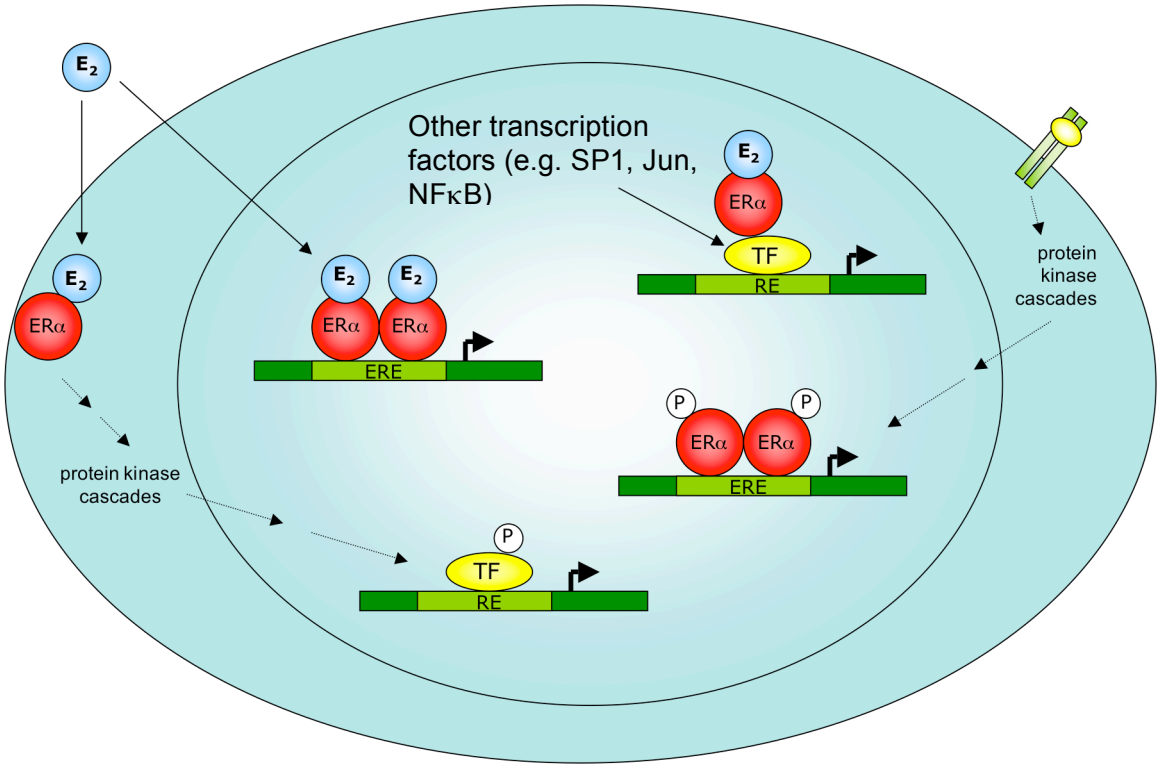


Figure 2. ER Signaling. ERα signaling pathways include: (1) genomic ERE-dependent ("classical"), in which liganded ER dimerizes and binds to an ERE; (2) genomic ERE-independent ("tethered"), in which liganded ER interacts with other transcription factors bound to their response elements; (3) membrane-initiated, in which a membrane-associated ER acts through kinases to phosphorylate other transcription factors; (4) ligand-independent, in which ER is activated by phosphorylation. Pathways 2-4 are collectively referred to as "non-classical".

necessary for the formation of protein-protein interactions and/or recruitment of co-activator or co-repressor molecules to the promoter regions of target genes.

iii. Rapid, Non-Genomic ER Signaling

Some of estrogen's effects have been shown to be so rapid that they cannot be attributed to activation of RNA and protein synthesis. Stimulation of estrogen-binding proteins localized to the plasma membrane can activate G-protein-coupled receptor signaling, second messenger generation (cAMP, cGMP), kinase and phosphatase activation, and calcium flux, and ultimately lead to functional changes at the cellular level (95). Several recent studies in a variety of cell types have identified these functional membrane proteins as the classical nuclear ERα (96-100), although other candidates for estrogen receptor proteins have been described, including GPR30 (101) and ER-X (102), among others (103, 104). Clearly the classical ERs are not transmembrane proteins, but they have been identified in membrane invaginations called caveolae (105-107) and may be G-protein-linked (100, 108). Importantly, rapid signaling and cell actions via membrane ERα have been described *in vivo*, including within the mouse brain (109, 110).

iv. Ligand-independent ER Signaling

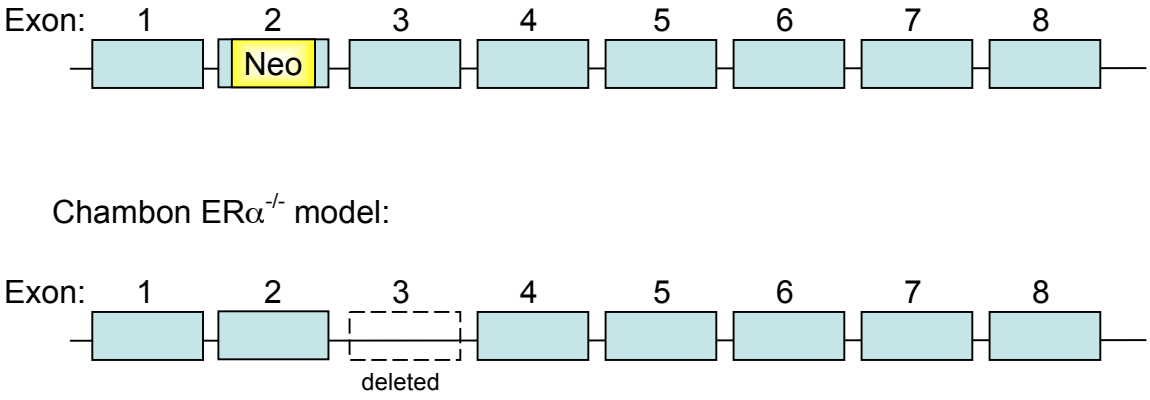
ER function can be modulated in the absence of E_2 by extracellular signals. For example, polypeptide growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) can activate ER and increase transcription of ER target genes (111, 112). In this pathway, gene activation occurs predominantly through second-messenger pathways that alter intracellular kinase and phosphatase activity, resulting in altered phosphorylation of ER (113).

d. Estrogen Receptor Knockout Mice

Mouse models of ER gene deletion were initially created using targeted mutagenesis (20, 114). In the first ERα knockout model, Korach and colleagues utilized the introduction of a Neo gene in the *Not*I site of exon 2 to disrupt the reading frame of the ERα gene (ERαKO) (Figure 3) (114). An estrogen-binding protein translated from an alternatively spliced transcript of the ERα gene, called E1, has been identified in several ERαKO tissues, including brain (115-118). As this splice variant is transcriptionally active, it remains a possibility that even low levels of expression may be sufficient to mediate estrogen's effects in target tissues. In 2000, Chambon and colleagues created a new ERα knockout model (which will be referred to as $ER\alpha^{-/-}$) that was generated by the complete deletion of exon 3, and thus represents a true null mutation (Figure 3) (119). It is this $ER\alpha^{-/-}$ model that was used exclusively in the present studies. Nonetheless, both knockout models have provided a wealth of information on the role of ERα in a number of physiological and behavioral systems.

Initial characterization of ERαKO males revealed that, although the reproductive tract develops normally during the prenatal period, these mice are completely infertile (16). ERαKO testes appear normal until puberty but begin to degenerate as early as 20-40 days of age and are atrophic by 150 days. As ERαKO males age, seminiferous tubules become atrophic due to back-pressure from luminal fluid retention (120), and sperm counts are significantly reduced (16). Epididymal sperm display reduced motility and some abnormal morphology, and even normal sperm were unable to fertilize wild-type oocytes in early *in vitro* fertilization assays (16). However, younger ERαKO males do produce viable sperm, suggesting that ERα deletion may not affect the germ cells themselves, but rather cause physical disruption of the testis during spermatogenesis. Accordingly, when ERαKO spermatogonia were implanted into the rete testes

A.



B.

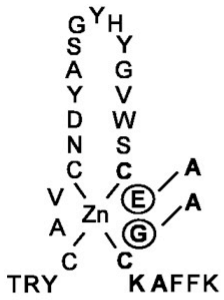


Figure 3. ERα gene knockout and knock-in models. (A) Schematic of ERα knockout strategies (not to scale). The ERαKO model created by Ken Korach and colleagues was generated by insertion of a Neo gene into exon 2, which disrupts the reading frame. The ERα$^{-/-}$ model created by Pierre Chambon and colleagues was generated by complete deletion of exon 3. In the ERαKO model, a small transcript variant encodes a truncated ERα protein that still possesses the DBD and LBD of wild-type ERα, as well as significant estrogen-dependent transcriptional activity. (B) The non-classical ERα knock-in mouse model. Point mutations introduced into mouse ERα created two amino acid substitutions in the P-box region of the first zinc-finger of the DNA binding domain. Changing E207 and G208 to alanine residues eliminates DNA binding while preserving protein structure. (Jakacka et al., 2002).

of wild-type host mice, they were able to successfully colonize the host testis, become functional sperm, fertilize oocytes, and produce viable offspring (121). This demonstrates that ERα is critical in the supporting somatic cell environment for normal sperm function but not in the germ cell.

The apparent infertility in ERαKO males is also attributed to severe deficits in sexual behavior (discussed in detail in section B.4 below) (16, 60). In contrast, ERβKO males are fertile, display no striking phenotypes, and display normal sexual behavior (20, 21), suggesting that male fertility depends largely, if not exclusively, on ERα.

e. Non-classical Estrogen Receptor Knock-In Mice

Recent evidence has revealed that ERα can participate in a number of non-classical signaling pathways that do not depend on direct receptor-DNA binding (described in section A.2.c above). Knock-in mouse models are one approach to dissecting multiple signaling activities *in vivo*. For example, mice expressing dimerization-deficient glucocorticoid receptor (GR), in which GR cannot bind to DNA, have been used to examine the roles of DNA binding-dependent and -independent GR signaling pathways in the hypothalamic-pituitary-adrenal axis (122). To better define non-classical signaling mechanisms of ERα action, Jakacka and colleagues generated selective DNA binding mutations in the mouse ERα and observed their effects *in vitro* (80). One such mutation within the first zinc finger of the DNA binding domain, E207A/G208A ("AA"), completely eliminated ERE binding and activation of ERE-containing reporter genes, but retained full transcriptional activity of reporter genes containing AP1 response elements and interacted with Jun when tested in mammalian cell two-hybrid assays (Figure 3). Of note, the AA mutant ERα protein has normal structure, equal expression and

activity compared to the wild-type protein, and does not exert dominant-negative effects *in vitro* (26). These findings demonstrated that ERα-DNA binding is not necessary for activity in the non-classical AP1 pathway.

This E207A/G208A mutation was introduced into the mouse ERα by targeted insertion (knock-in) in order to distinguish between classical and non-classical ERα actions *in vivo* (26). Initial characterization of the resulting $ER\alpha^{+/AA}$ mice revealed that females are acyclic and display reduced serum progesterone, anovulation, uterine defects, and inhibited mammary gland development. These results are surprising given that $ER\alpha^{+/-}$ females are fertile (114, 119), and therefore suggest a putative antagonism of the wild-type allele by the AA mutation, or an imbalance of the relative activities of classical and non-classical pathways. Nonetheless, the phenotype of these heterozygous $ER\alpha^{+/AA}$ mice demonstrates the physiological importance of non-classical ERα signaling in the development and function of the female reproductive system.

Unfortunately, the resulting infertility of $ER\alpha^{+/AA}$ females precludes the generation of $ER\alpha^{AA/AA}$ homozygous mutants. However, $ER\alpha^{+/AA}$ males appear to have normal fertility (26) and can therefore be used to generate $ER\alpha^{-/AA}$ compound heterozygotes when bred with $ER\alpha^{+/-}$ females. Introducing the AA knock-in mutation on the ERαKO background effectively eliminates all ERE-dependent signaling, therefore a rescue of $ER\alpha^{-/-}$ phenotypes by the AA mutation suggests a physiological role for non-classical mechanisms. Thus, these $ER\alpha^{-/AA}$ mice provide a unique opportunity to examine isolated ERE-independent signaling. As such, they have been successfully used to identify a physiological role for non-classical ERα signaling in uterus (123), bone (124), and estrogen feedback on LH secretion in the female (125).

To date, there have been no studies that examine the role of ERE-independent ERα signaling pathways in male physiology and behavior *in vivo*. Preliminary examination of ERα$^{-/AA}$ male mice revealed a transient rescue of the tubule dysmorphogenesis in the testis that results from ERα deletion, suggesting that non-classical ERα signaling pathways may contribute to estradiol regulation of male reproductive function (Jeffrey Weiss, unpublished observations). The purpose of the studies presented in Chapters III and IV was therefore to examine the relative roles of classical and non-classical ERα signaling in male sexual behavior and hormone production.

B. Male Sexual Behavior

1. Steroid Regulation

It is well known that testosterone and its metabolites are critical for the central control of male sexual behavior. Upon surgical castration, sex drive and sexual activity are greatly, if not completely, diminished, but can be restored to pre-castration levels by T replacement (126). Following castration, male rats may continue to exhibit sexual behavior for days or weeks, despite the fact that plasma T levels fall to immeasurable levels within 24 hours (126, 127). While low doses of T are sufficient to maintain copulatory behavior when the hormone is administered soon after castration, higher doses are required if behavior has been completely lost (126). This may suggest that continuous exposure to steroids maintains responsiveness of neural and peripheral tissues, and that after a prolonged absence a greater input is needed to stimulate activity. The amount of time required to restore behavior to long-term castrates with T (days to weeks) is also consistent with slow, genomic mechanisms (126, 128-132). The importance of

genomic actions of steroids is further supported by the findings that the protein synthesis inhibitor anisomycin blocked sexual behavior in male rats (133).

T metabolites estradiol and DHT are differentially effective in stimulating copulation. In castrated male rats, E_2 is both necessary and sufficient to maintain or restore most aspects of behavior (134-136), and aromatase inhibitors or ER antagonists block the restoration of behavior by T (137-139). The aromatase inhibitor fadrazole also inhibited sexual behavior in gonadally intact male rats (140). Synthetic androgens that can be metabolized to E_2 but not DHT are also extremely effective in restoring sexual behavior to castrated rats (141) and mice (142). The importance of E_2 and ER signaling is further supported by studies demonstrating deficits in male sexual behavior in ER knockout mice (discussed in detail in section B.4 below).

Importantly, E_2 administration alone is not sufficient to maintain ejaculation (137, 140, 143-146), and studies using AR agonists and antagonists suggest that AR stimulation does contribute to T's effects (130, 146, 147). DHT alone is not sufficient to restore copulation in castrated rats (148, 149), but is sufficient to restore sexual behavior in other species, such as mice (150). Conversion of T to DHT may be essential for maintenance of peripheral sensory and motor functions, and DHT is fully effective in maintaining or restoring penile reflexes (151-154). E_2 and DHT administered together are effective in restoring the full complement of copulatory behavior (155, 156), demonstrating that stimulation of both ER and AR is important for the activation of male sexual behavior.

2. Brain Regions Controlling Male Sexual Behavior

Early studies attempting to localize the effect of T in the brain identified the medial preoptic area (MPOA) as being the most effective site for the restoration of copulation in

castrated male rats (157, 158). The MPOA is now known to be a critical integrative component of the neural organization of copulation in all male vertebrate species that have been tested, as it receives input from, and sends reciprocal connections to, every sensory source (159). Accordingly, lesions of the MPOA completely abolish and electrical stimulation facilitates copulation in a number of species (3). However, it is important to note that local drug or steroid implants are often less effective than systemic treatments, suggesting that activating a limited portion of the neural circuitry involved in behavior may not be sufficient for the full maintenance or restoration of behavior in castrates (134, 157, 160, 161). Indeed, several other brain regions contribute to the expression of copulatory behavior, including the olfactory bulb, amygdala, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, ventromedial hypothalamus, and lateral hypothalamus, among others (3).

3. Neuronal Activity

The integration of neuronal and endocrine events plays a major role in the regulation of male sexual behavior. Electrophysiological studies demonstrate that castration decreases and T increases the spontaneous firing rate of neurons in the MPOA and anterior hypothalamus. Infusion of T restores the firing rate and increases the neural sensitivity to sensory and electrical stimulation (5). Thus, increased excitability may render neurons more sensitive to the hormonal, chemical, and somatosensory inputs they receive, presumably increasing the likelihood of producing a steroid-dependent behavioral response. Additional evidence indicates a direct effect of castration and T replacement on membrane properties of neurons involved in the control of male sexual behavior, such as the absolute refractory period, the interval during which a second action potential absolutely cannot be initiated, no matter how large a stimulus is applied (4, 6).

However, relatively little is known about the molecular mechanisms by which T alters neural activity to elicit male sexual responses. It is generally believed that T exerts its effects on male sexual behavior via slow, genomic actions (126, 133, 136), and the similar, long-term time courses for the behavioral and electrophysiological effects of T suggest a common underlying mechanism (162). However, it is not known how T exerts these effects on central neurons.

a. ATP-sensitive Potassium Channels

Recent evidence suggests that regulation of inwardly rectifying potassium ion channels may determine cell excitability. Inward rectifiers primarily function to stabilize resting membrane potential and mediate potassium flux across membranes, thereby modulating the electrical activity of cells (163). One particular type of inwardly rectifying potassium channels, ATP-sensitive potassium (K^+_{ATP}) channels, were first discovered on the cell surface of pancreatic β-cells (164), and were subsequently identified in the heart (165), blood vessels (166), and brain (9). Autoradiography studies (167-170), *in situ* hybridization (7, 10, 171, 172), and electrophysiological techniques (173) have identified K^+_{ATP} channels in a variety of neuronal populations. In the central nervous system, K^+_{ATP} channels maintain resting membrane potential at more negative levels (approximately -65mV) (8), mediate glucose-sensing (31), regulate neurotransmitter release (9, 13, 14), and provide neuroprotection against seizure activity (174).

The K^+_{ATP} channel is a heterodimer of four inwardly rectifying subunits (Kir6.x) and four high-affinity sulfonylurea receptor subunits (SUR) (Figure 4). In neuronal tissue, functional channels are predominantly composed of the Kir6.2 and SUR1 subunit isoforms (7), which exhibit a 1:1 stoichiometry (175). K^+_{ATP} channels conduct potassium ions into the cell, switching quickly between open and closed states. Closure of K^+_{ATP} channels blocks membrane

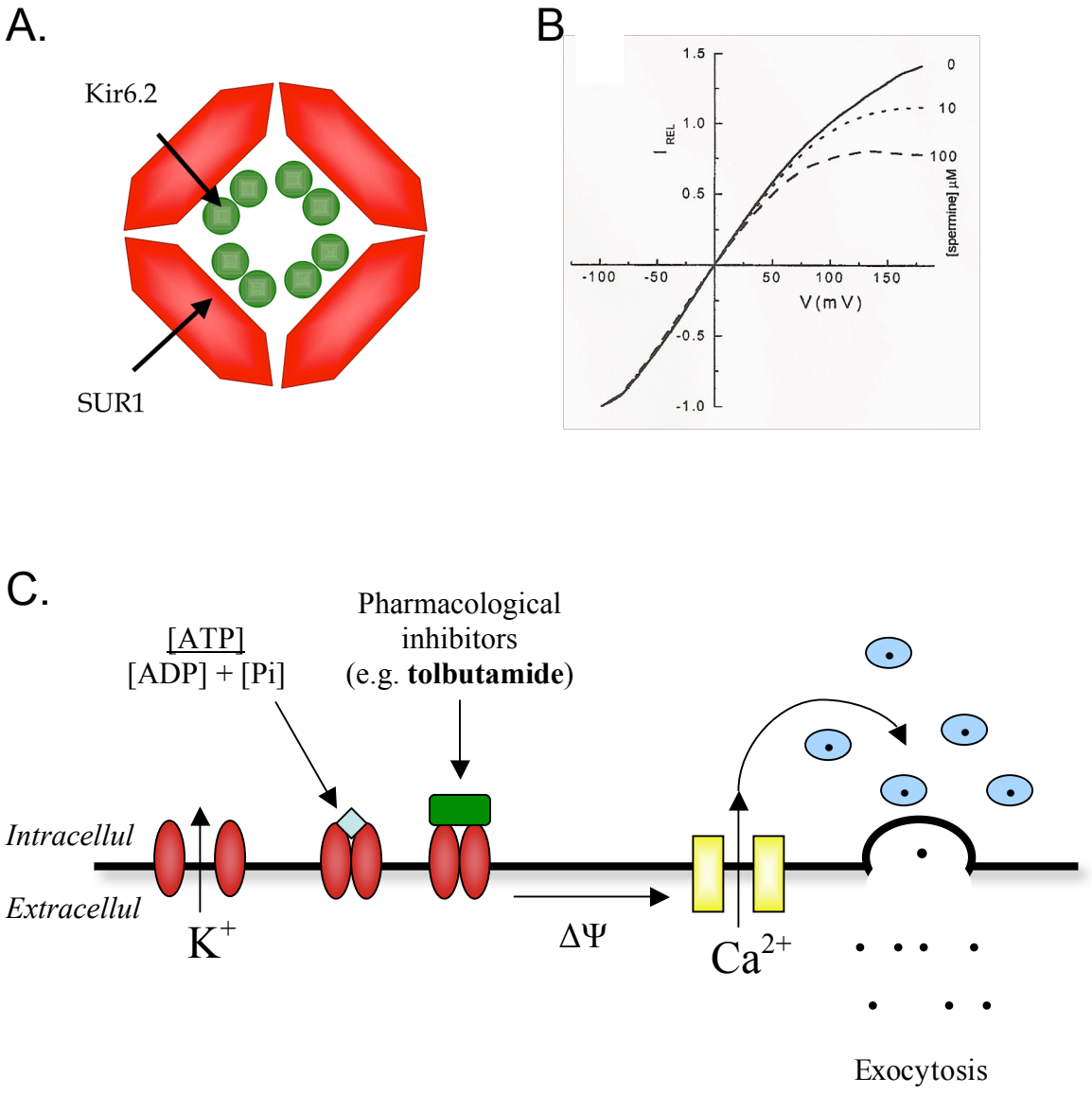


Figure 4. ATP-sensitive potassium channels. (A) Neuronal K^+_{ATP} channels are heterodimers of four inwardly rectifying Kir6.2 subunits and four high-affinity sulfonylurea receptor SUR1 subunits. (B) A current vs. voltage plot to show the inward rectification (reduced current at more positive potentials) of K^+_{ATP}channels. The effect of an added polyamine, spermine, is also shown. (Aguilar-Bryan and Bryan, 1999) (C) K^+_{ATP} channels conduct potassium ions into the cell. Closure of K^+_{ATP} channels by ATP or pharmacological inhibitors such as tolbutamide blocks membrane permeability to K^+ and shifts the membrane potential to more depolarized levels. Depolarization of the membrane induces the opening of voltage-gated Ca^{2+} channels and allows Ca^{2+} to enter the cell, increasing intracellular Ca^{2+} concentrations. This causes calcium-dependent exocytosis and secretion (e.g. insulin secretion from pancreatic β-cells, neurotransmitter release from neuron synapses.)

permeability to K^+ and shifts the membrane potential to more depolarized levels. Depolarization of the membrane induces the opening of mainly L-type voltage-gated Ca^{2+} channels (at an activation threshold of approximately -30mV) and allows Ca^{2+} to enter the cell, increasing intracellular Ca^{2+} concentrations. This subsequently causes calcium-dependent exocytosis and secretion, such as insulin secretion in pancreatic β-cells and neurotransmitter release from neuron synapses. Conversely, opening of K^+_{ATP} channels results in increased permeability, hyperpolarization, decreased cell excitability, and therefore reduced secretion (7).

Binding of ATP or channel modulators to the K^+_{ATP} channel inhibits its activity. For example, the pharmacological agent tolbutamide specifically binds to the sulfonylurea binding site on the SUR1 subunit and closes the channel (7). In contrast, the potassium channel opener diazoxide strongly activates Kir6.2/SUR1 channels (176). Similar to closure of K^+_{ATP} channels, a decrease in expression of functional channels is believed to shift the membrane potential to more depolarized levels and increase cell excitability, whereas an increase in expression hyperpolarizes the cell and dampens cell excitability. In support of this idea, overexpression of SUR1 in the forebrain renders mice resistant to seizures and excitotoxic neuronal death (177).

Currently, the role of these channels as targets of hormone action and in sexual behavior is not well defined. Using *in situ* hybridization and immunocytochemistry, Dunn-Meynell and colleagues observed that cells containing Kir6.2 mRNA were abundant in brain regions involved in sexual behavior, including the medial preoptic nucleus, antero-medial hypothalamus, medial amygdala, and lateral septum, among others (10). In sexually experienced, intact male rats systemic administration of the K^+_{ATP} channel blocker glibenclamide increased male sexual behavior, however, it is not clear if this was due to a central or peripheral effect (178). Regulation of K^+_{ATP} channel subunit expression has been previously demonstrated for glucose

(179), glucocorticoids (12), and estrogen and progesterone (11), but it is not known whether these channels are regulated by testosterone in neural tissue.

4. Molecular Actions of Estrogen in Male Sexual Behavior

a. Estrogen Receptor Alpha vs. Estrogen Receptor Beta

E_2 clearly plays a role in the regulation of male sexual behavior (as discussed in section B.1 above), and steroid receptor gene knockout mice have been valuable resources for examining the molecular mechanisms of E_2 action. Initial characterization of male ERαKO mice demonstrated that they were unable to produce offspring in a continuous mating study with wild-type, known-fertile females (16). Subsequent behavioral analyses of ERαKO males revealed that intact and T-treated castrates displayed mounting behavior, albeit with longer latency and lower frequency, but very few intromitted and none ejaculated (17, 18, 180). Sexual attraction toward wild-type females initially appeared to be normal, but specific tests of sexual motivation and social preference indicate that ERα is necessary for sex discrimination and the motivational aspects of sexual behavior (17, 18, 181). Interestingly, the profound defects in sexual behavior and social preferences in ERαKO males have been shown to be restored by systemic treatment with the dopamine agonist apomorphine, suggesting that the neural circuitries governing behavior are sufficiently organized in the absence of ERα and that ERα may function by stimulating the neural release of dopamine (18).

In contrast to ERαKO males, ERβKO males have been shown to be no different than wild-type males in the display of masculine copulatory behavior (21). However, behavioral puberty in ERβKO males is significantly delayed (22) and castrated adult ERβKO males treated with estrogen and progesterone showed high levels of female-typical sexual receptivity,

indicating incomplete defeminization of the brain and behavior (23). The complete absence of sexual behavior observed in ERαβKO males led Ogawa and colleagues to conclude that either one of the ERs is sufficient for the expression of mounting in male mice, indicating a redundancy of function (182).

When evaluating behavioral data in mice, strain effects must be taken into consideration as genetic variability clearly contributes to differences in behavior (183, 184). Steroid receptor knockout mice are initially of mixed stock (129/SV and C57Bl/6) and are usually backcrossed onto the C57Bl/6 background into a single inbred line. Of note, the original work describing sexual behavior of ERαKO and ERβKO males was conducted on mice of a mixed 129/SV and C57Bl/6 background (16, 71) and some early findings are at odds with those using mice that have been backcrossed onto C57Bl/6, namely that mounting and intromissions were reported in mice of mixed backgrounds while significantly lower rates were noted in backcrossed ERαKOs (185). Furthermore, when backcrossed ERαKO males were outbred to either BALB/c or DBA/2J strains, several male ERαKOs displayed significantly improved intromissive behavior, some attained ejaculation, and one animal outcrossed to DBA/2J sired a litter (186). Thus, the normal mounting behavior initially reported in ERαKO males may reflect a more robust behavioral phenotype due to their mixed background.

While these studies of ERαKO mice clearly indicate the importance of ERα in copulation and other sexually motivated behaviors, the exact cellular and molecular mechanisms of E_2 action are not defined.

b. Classical vs. Non-classical Mechanisms of Estrogen Signaling

As mentioned previously, slow, genomic mechanisms and protein synthesis appear to be necessary for steroid-induced sexual behavior (133). However, other evidence demonstrates that rapid, non-genomic effects of E_2 additionally contribute to male sexual behavior. For example, E_2 rapidly affects ion channels in neuron membranes (95, 110, 187-190), alters neuronal firing within minutes in male preoptic area slices (191), and rapidly stimulates copulation in castrated rats (192), castrated quail (193), and aromatase knockout mice (194). However, E_2-induced recovery of sexual behavior may rely on sub-threshold doses of or recent exposure to steroids, suggesting that slow, genomic actions of T and/or E_2 may prime the neural mechanisms that confer sensitivity to E_2's rapid actions. Indeed, evidence suggests that there may be integration of non-genomic and genomic pathways (195, 196) and that E_2 stimulates male sexual behavior through a combination of mechanisms (197). It is also possible that the short-term actions of E_2 exert minor, facilitating effects, such as increasing sensitivity to sensory cues, which are separate from the major genomic stimulus that is required to initiate sexual behavior. Whether the rapid actions of E_2 are specifically mediated by a membrane-associated ERα remains to be determined.

5. Measuring Male Sexual Behavior

Rodent models have long been used to investigate the hormonal, neuronal, and sensory contributions to sexual activity. The rat is perhaps the most widely used model, largely due to the well-defined stereotypic behaviors displayed by both sexes, as well as its frequent use in neurobiology and endocrinology in general. In the laboratory setting, males are paired with sexually receptive stimulus females and allowed to copulate freely during a given time period.

The pattern of sexual behavior typically begins with paracopulatory behaviors, such as anogenital sniffing, and then, upon assessing the female's receptivity, a male will attempt to mount by lifting his forebody over the female's hindquarters, clasping her flanks with his forepaws, and performing a series of rapid and shallow pelvic thrusts. A receptive female will remain immobile during this investigation and mounting and will often display lordosis posture, which gives the male greater access to the vaginal opening. If the male detects the vaginal opening and successfully achieves penile insertion, the pelvic thrust is deeper and is termed an intromission. Intromissions are also characterized by a springing dismount and subsequent genital grooming. After a series of intromissions, a male may achieve ejaculation, which is distinguished by a slightly longer intromissive thrust or "freezing", followed by a slower, relaxed dismount. A period of sexual quiescence then follows male ejaculation. While sexual behavior of male mice is essentially the same as in the rat, intromissions are more rhythmic and do not involve a springing dismount, and ejaculation is typically displayed by a more pronounced freezing and clasping of the female. I have utilized these standard behavioral testing techniques in the present studies to investigate the effect of a K^+_{ATP} channel inhibitor on male sexual behavior in the rat, and to investigate the effect of non-classical ERα signaling on male sexual behavior in the mouse.

C. The Hypothalamic-Pituitary-Gonadal Axis

1. Overview

Normal testis function, and thus steroidogenesis and spermatogenesis, is tightly controlled within the hypothalamic-pituitary-gonadal (HPG) axis. The pulsatile release of gonadotropin-releasing hormone (GnRH) is critical for sustaining a cascade of hormone

secretions. GnRH, synthesized in preoptic and periventricular neurons, is transported neuronally to the median eminence, and released into the hypothalamic-hypophysial portal vessels in a pulsatile manner. It then binds to plasma membrane receptors on gonadotropes in the anterior pituitary, stimulating the synthesis and episodic pulsatile secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH enter systemic blood and act on the gonads to stimulate steroid hormone secretion and spermatogenesis.

2. Negative Feedback

Testosterone released from the testes acts back on the HPG axis through a tonic negative feedback system. In the male, castration results in substantially elevated circulating levels of LH, which are restored by testosterone administration (198). T also inhibits FSH secretion, but complete suppression comes from the combined action of T and the testicular peptide hormone inhibin (199). The observed increase in LH pulse frequency following castration is attributed to an effect on the hypothalamus, while an increase in LH pulse amplitude reflects a change in responsiveness of the pituitary gonadotropes to GnRH. Accordingly, a number of studies have demonstrated that castration and steroid replacement alter levels of GnRH mRNA (200, 201), processing of GnRH prohormone (202-204), hypothalamic GnRH contents (205-209), and patterns of pulsatile GnRH release (210-212). At the level of the pituitary, T affects responsiveness to GnRH (198, 211, 213), gonadotropin secretion (214), and gonadotropin expression (215).

The exact mechanisms of negative feedback on GnRH and gonadotropin secretion in the male are still largely unknown. AR-mediated mechanisms are supported by evidence that DHT treatment is capable of suppressing serum LH and steady-state levels of gonadotropin subunit

mRNA (215-217). Furthermore, blockade of AR with the antagonist flutamide results in elevations of serum LH (218). Direct androgen action is also supported by the presence of ARs in gonadotropes and nuclei in the arcuate nucleus-median eminence of the hypothalamus (37, 219). However, early immunocytochemical and autoradiography studies failed to demonstrate the expression of ARs in GnRH neurons themselves (220, 221). Although appreciable amounts of AR have yet to be localized on GnRH neurons *in vivo*, functional receptors have been observed in the immortalized, GnRH-producing GT_1-7 cells (222). Thus, it is unclear whether AR-mediated androgen negative feedback occurs directly on GnRH neurons.

Some studies have suggested that T's negative feedback effects are mediated at least in part by ER following T's aromatization to E_2. For example, ERα and ERβ mRNAs have been identified in GnRH neurons using single-cell PCR (223), and the presence of ERβ protein on GnRH neurons has also been well established through immunocytochemistry, but the presence of ERα still remains slightly controversial (224-228). There is also some debate about the role of ERα in estrogen negative feedback on LH, based on evidence from the ERαKO mouse model. For example, some studies have shown that ERα deletion does not result in a consequent rise in LH (16, 229), while other findings suggest that, although hypothalamic GnRH contents can be maintained solely through AR signaling pathways, normal regulation of gonadotrope function requires activation of ERα (230). It is worth noting that the approximately 2-fold greater LH levels reported in intact ERαKO males are still not as high as those in castrated wild-type males. Moreover, castration of ERαKO males resulted in a significant rise in LH, suggesting that negative feedback on LH by T may be mediated at least in part by mechanisms independent of ERα (230). Clearly ERα is critical for negative feedback in the female, as demonstrated by extremely high LH levels in ERαKO female mice (231); however, the androgen receptor, or

perhaps ERβ, may play the predominant physiological role in the male (229). Therefore, one objective of these studies is to further define the involvement of ERα in estrogen negative feedback in the male.

3. Androgen Biosynthesis

a. Leydig Cell Function

The interstitial tissue of the testis makes up 5% of the total testicular volume and contains the primary endocrine cells of the testis, the Leydig cells. Leydig cells are the main source of T synthesis and secretion in the male and are under direct control of pituitary LH. LH binds to receptors on the cell surface and stimulates cAMP production, which activates the steroidogenic pathway. Leydig cells produce T for systemic transport into the blood stream and also initiate paracrine interactions with adjacent seminiferous tubules and Sertoli cells to influence spermatogenesis.

In the first, rate-limiting step of steroid biosynthesis, a specific cholesterol transport protein known as steroidogenic acute regulatory protein (StAR) facilitates the delivery of cytoplasmic cholesterol substrate across the outer mitochondrial membrane to the inner mitochondrial membrane (Figure 5). Once in the mitochondria, cholesterol is converted to pregnenolone by the P450 side chain cleavage enzyme. Subsequent conversion of pregnenolone to T requires four enzymatic reactions, which are divided into two parallel pathways: the Δ^5-pathway (via 17-hydroxypregnenolone) and the Δ^4-pathway (via progesterone). The relative activities of these pathways differ between species, with the Δ^4-pathway being predominant in rodents and the Δ^5-pathway being predominant in humans. These enzymatic reactions include conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase, conversion of

progesterone to 17α-hydroxyprogesterone by P450 17α-hydroxylase/$C_{17–20}$ lyase, and conversion of androstenedione to T by 17β-hydroxysteroid dehydrogenase (Figure 5). The relative abundance and activities of these steroidogenic enzymes ultimately determines the amounts of end product produced.

Estradiol is produced within the Leydig cells by P450 aromatase, however, it is also produced in the brain, liver, fat, and skin. DHT is produced by 5α-reductase in Leydig cells, but it is formed predominantly in target tissues such as skin, submaxillary glands, and prostate.

b. Estrogen Regulation

Endogenous estrogens are clearly necessary for maintaining normal reproductive function, as evidenced by the negative effects resulting from estrogen deficiency. Men deficient in the estrogen-producing enzyme aromatase (232), aromatase knockout mice (233, 234), and ERαKO mice all display high levels of serum T. Indeed, Leydig cells express both ERα and ERβ and are thus sensitive to estrogen action (65, 235, 236). It is now known that endogenous estrogens inhibit T production during both fetal and neonatal development (237) and in adulthood (25, 238). Furthermore, treatments with exogenous estrogens or estrogenic compounds have been shown to decrease serum T and intratesticular T content *in vivo* (235, 239-246), reduce Leydig cell T secretion *in vitro* (247-250), inhibit Leydig cell regeneration (251), and suppress expression of steroidogenic enzymes (252, 253).

While serum T levels are significantly elevated in the ERαKO male mouse (16, 25, 229, 254, 255), they are normal in ERβKO males (20). Estrogen treatments effectively reduce serum T in WT males but not ERαKO males, indicating that the presence of ERβ in ERαKO testes is

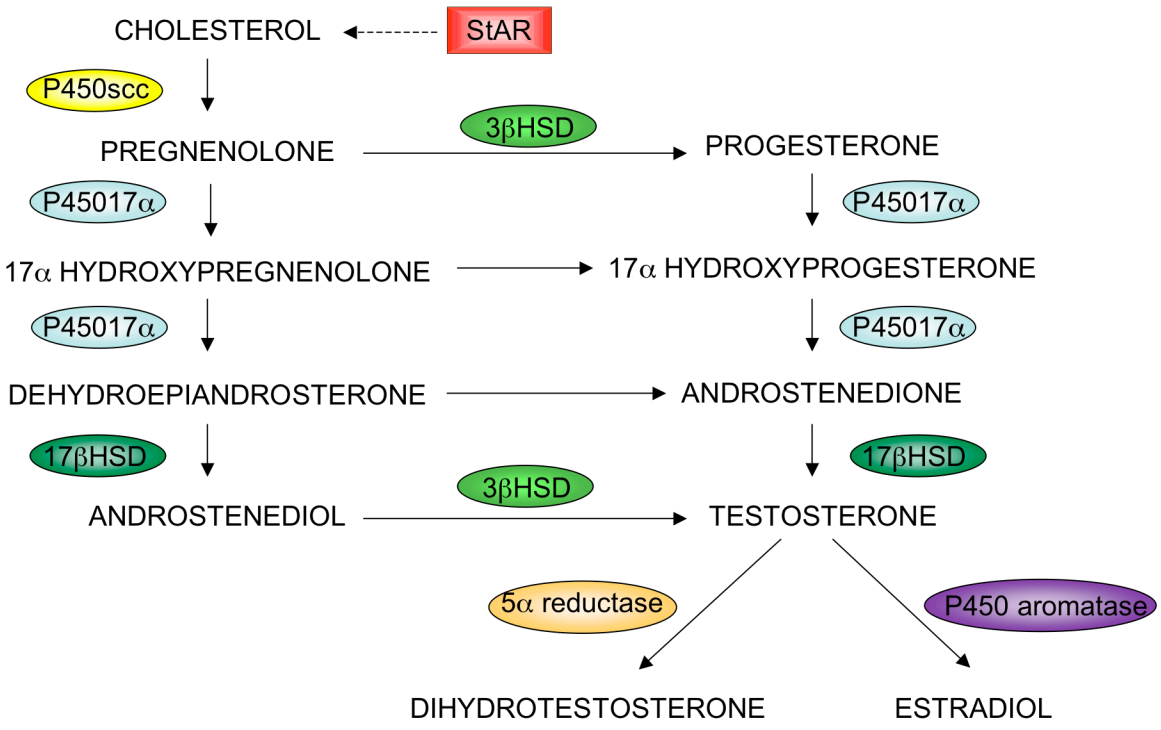


Figure 5. Testosterone synthesis and metabolism. In the first, rate-limiting step of steroid biosynthesis, StAR facilitates the delivery of cholesterol substrate across the outer mitochondrial membrane to the inner mitochondrial membrane. Once in the mitochondria, cholesterol is converted to T through a series of enzymatic reactions, which are divided into two parallel pathways: the Δ^5-pathway (via 17-hydroxypregnenolone) and the Δ^4-pathway (via progesterone). The Δ^4-pathway is predominant in rodents and the Δ^5-pathway is predominant in humans. Testosterone can be metabolized to DHT by the enzyme 5α-reductase or estradiol by the enzyme aromatase.

not sufficient to mediate estrogenic actions on T secretion (25). These findings demonstrate that estrogen inhibition of androgen biosynthesis is mediated largely, if not solely, through ERα.

It remains somewhat controversial whether increased serum T levels in ERαKO males are the direct result of increased steroidogenesis or the indirect result of disrupted negative feedback on gonadotropin secretion, and thus increased stimulation by pituitary LH. While some studies have reported moderately increased LH levels in adult ERαKO male mice, others fail to see an effect of genotype on serum LH (as discussed in section C.2 above) (16, 229, 255). Evidence from fetal ERαKO mice, in which LH levels have not begun to exert stimulation on the testis, and neonatal ERαKO males, in which no change in circulating LH was observed, suggest a direct inhibitory effect of endogenous estrogens (254). In rams, low level estradiol immunoneutralization also induces elevations in serum T, increased StAR mRNA levels, and increased 17β-HSD activity without affecting pituitary LH secretion (256). Furthermore, studies using adult Leydig cell cultures from gonadotropin-deficient mice reveal that regulation of 3β-HSD expression is independent of LH stimulation (257). These findings support the idea of a direct LH-independent effect of estrogen signaling on Leydig cell steroidogenesis (25).

Estrogen clearly exerts effects through a number of ER signaling pathways. Interestingly, a G-protein-coupled estrogen receptor was shown to be present in the adult testis (258), and estrogen has been shown to suppress expression of P450 steroidogenic enzyme genes indirectly via inhibition of SF1 expression (253, 259). Moreover, evidence suggests that estrogens and xenoestrogens inhibit androgen production via a non-genomic action mediated by an estrogen membrane receptor in Atlantic croaker (260). However, it remains to be determined whether estrogens specifically inhibit androgen biosynthesis via classical, ERE-dependent or non-classical, ERE-independent ERα mechanisms in male mice.

D. Questions for Thesis Studies

Cellular mechanisms of T stimulation of sexual behavior

It is currently unknown how T enhances cell excitability in brain regions involved in male sexual behavior. Inwardly rectifying potassium ion channels contribute to cell excitability, and are particularly attractive candidates as mediators of T action because they primarily function to stabilize resting membrane potential and mediate potassium flux across membranes, thereby modulating the electrical activity of cells (163). I propose that androgens enhance neuronal excitability, and subsequently sexual responsiveness, by regulating neuronal potassium ion channels. Based on recent studies from my laboratory demonstrating steroid-regulated expression of ATP-sensitive potassium (K^+_{ATP}) channels in the female brain (11), I chose to investigate this particular inwardly rectifying potassium channel as a mediator of steroid-dependent male sexual behavior. I also chose to investigate K^+_{ATP} channels as mediators of T-dependent behavior based on evidence that these channels regulate cell excitability, modulate neurotransmitter release, and are present in brain regions that are involved in male sexual behavior (9, 10, 13, 14, 172). I proposed that T inhibits the expression of K^+_{ATP} channels, as a decrease in channel expression would increase cell excitability and thereby render neurons more sensitive to the hormonal, chemical, and somatosensory inputs they receive, thus increasing the likelihood of producing a behavioral response (Figure 6). Therefore, experiments in Chapter II use RT-PCR to determine if T inhibits expression of K^+_{ATP} channel subunits Kir6.2 and/or SUR1 in brain regions involved in male sexual behavior, such as the preoptic area and hypothalamus. As closure of channels would also increase cell excitability, pharmacological experiments in Chapter II were conducted to determine if K^+_{ATP} channel blockade affects male sexual behavior,

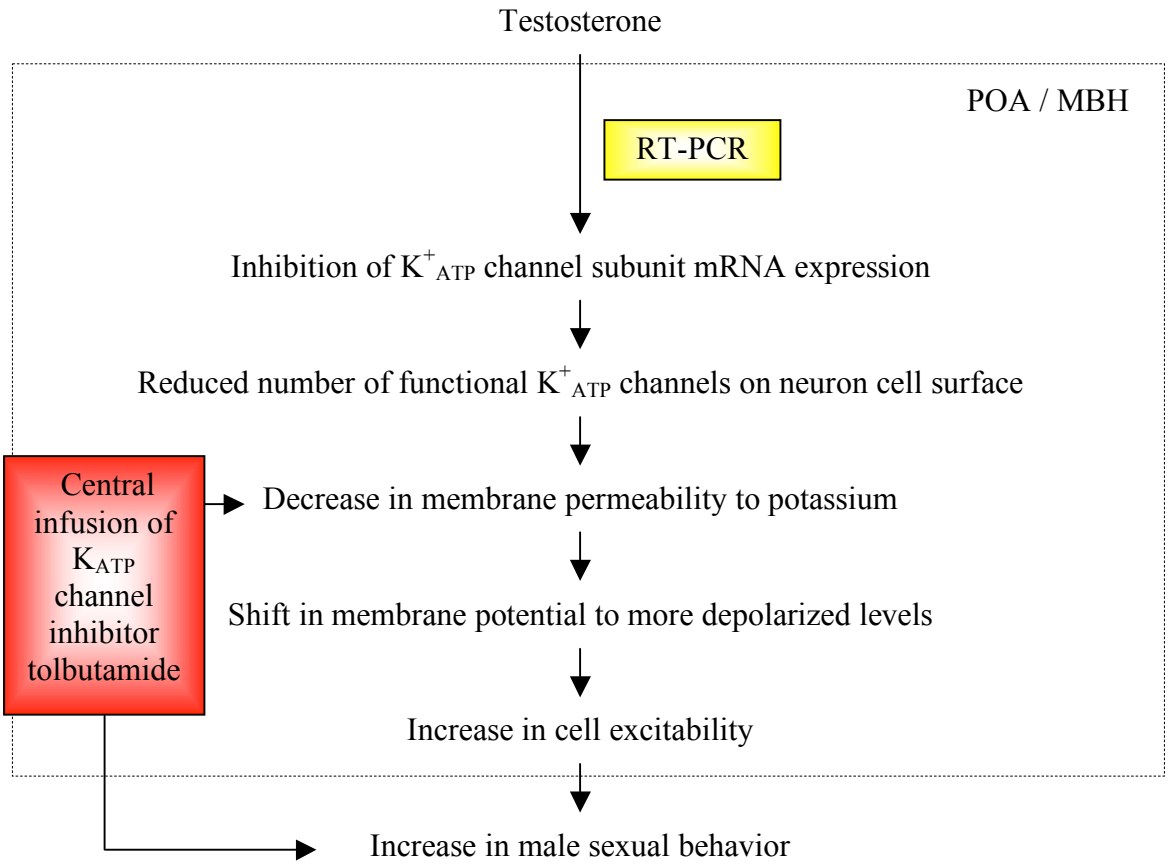


Figure 6. Proposed model for K^+_{ATP} channels as mediators of T-dependent male sexual behavior. Inhibition of K^+_{ATP} channel subunit expression and pharmacological blockade of K^+_{ATP} channels in the preoptic area (POA) and/or medial basal hypothalamus (MBH) would decrease membrane permeability to potassium, shift membrane potential to more depolarized levels, increase cell excitability, and increase the probability of sexual responsiveness. Therefore, experiments in Chapter II were designed to examine the effects of T on mRNA expression of K^+_{ATP} channel subunits Kir6.2 and SUR1 using RT-PCR and the effect of central infusion of the K^+_{ATP} channel inhibitor tolbutamide on copulation in male rats.

and specifically addressed the question: Can central infusions of the pharmacological K^+_{ATP} inhibitor tolbutamide improve sexual behavior of intact or castrated male rats?

Molecular mechanisms of T stimulation of sexual behavior

While it is clear that ERα is important for male sexual behavior, it remains to be determined whether non-classical, ERE-independent ERα signaling mechanisms mediate estrogen stimulation of copulation. My objective was to determine the relative contributions of classical and non-classical ERα signaling to male sexual behavior using the $ER\alpha^{-/AA}$ mouse model. Recovery of the $ER\alpha^{-/-}$ phenotype (i.e. little to no male sexual behavior) by the AA mutation in $ER\alpha^{-/AA}$ males would suggest that ERE-independent signaling mechanisms are sufficient to mediate estrogen's effects on sexual activity, whereas no recovery would indicate that ERE-dependent mechanisms are required. Experiments in Chapter III were therefore designed to test the hypothesis that non-classical ERα mechanisms can restore the deficits in male sexual behavior that result from ERα deletion. Specifically, these experiments addressed the question: Do $ER\alpha^{-/AA}$ male mice perform better than $ER\alpha^{-/-}$ males in tests for copulatory ability?

Molecular mechanisms of T regulation of the reproductive axis

In addition to examining the molecular mechanisms of ERα signaling in sexual behavior, I investigated whether non-classical, ERE-independent ERα signaling mechanisms also mediate estrogen regulation of the reproductive axis using the $ER\alpha^{-/AA}$ mouse model. Experiments in Chapter IV were conducted to determine serum T and gonadotropin levels in $ER\alpha^{-/AA}$ and $ER\alpha^{-/-}$ male mice. I hypothesized that non-classical ERα mechanisms could restore the enhanced T

biosynthesis that results from ERα deletion. Chapter IV specifically addressed the questions: Is T secretion rescued in $ER\alpha^{-/AA}$ testes? Can the knock-in mutation mediate E_2 suppression of steroidogenic enzyme gene expression or steroidogenic enzyme activity? Recovery of the $ER\alpha^{-/-}$ phenotype (i.e. high serum T levels, increased expression and activity of steroidogenic enzymes) by the AA mutation in $ER\alpha^{-/AA}$ males would suggest that ERE-independent signaling mechanisms are sufficient to mediate estrogen inhibition of steroidogenesis, whereas no recovery would indicate that ERE-dependent mechanisms are required.

CHAPTER II: A ROLE FOR ATP-SENSITIVE POTASSIUM (K^+_{ATP}) CHANNELS IN MALE SEXUAL BEHAVIOR

Abstract

ATP-sensitive potassium (K^+_{ATP}) channels regulate cell excitability and are expressed in steroid-responsive brain regions involved in sexual behavior, such as the preoptic area (POA) and medial basal hypothalamus (MBH). I hypothesized that K^+_{ATP} channels serve as a mechanism by which testosterone can control the electrical activity of neurons and consequently elicit male sexual responsiveness. RT-PCR analysis indicated that castration induces, while testosterone inhibits, mRNA expression of the K^+_{ATP} channel subunit Kir6.2 in both the POA and MBH of adult male rats. Intracerebral infusion of the pharmacological K^+_{ATP} channel inhibitor tolbutamide increased the proportion of long-term castrates displaying sexual behavior and restored mount frequency, intromission frequency, and copulatory efficacy to values observed in testes-intact animals. Infusions of tolbutamide, but not vehicle, also decreased latencies to mount and intromit in castrated males. Unilateral tolbutamide infusion directly into the POA significantly reduced mount latency of castrates; however, it did not affect other copulatory measures, suggesting that blockade of K^+_{ATP} channels in additional brain regions may be necessary to recover the full range of sexual behavior. These data indicate that blockade of K^+_{ATP} channels is sufficient to elicit the male sexual response in the absence of testosterone. My observations are consistent with the hypothesis that testosterone stimulates male sexual behavior by inhibiting K^+_{ATP} channels in the brain. Decreased channel expression or channel blockade may increase the excitability of androgen-target neurons, rendering them more sensitive to the hormonal, chemical, and somatosensory inputs they receive, and potentially increase secretion of neurotransmitters that facilitate sexual behavior.

Introduction

Central testosterone (T) action is critical for male sexual behavior. However, the cellular and molecular actions underlying T's influence on neuronal activity and sexual responsiveness are not well defined. Electrophysiological studies demonstrate that infusions of T into the medial preoptic area and anterior hypothalamus of castrated rats increase the spontaneous firing rate of neurons and increase the neural sensitivity to sensory and electrical stimulation (5). Additional evidence demonstrates a direct effect of castration and T replacement on membrane properties of neurons involved in the control of male sexual behavior (4, 6). While there is evidence to suggest that steroids can have rapid effects on neuronal activity and copulation (5, 191-194, 261), it is generally believed that T exerts its effects on male sexual behavior via slow, genomic actions (126, 133, 136). The similar time courses for the behavioral and electrophysiological effects of T may suggest a common underlying mechanism (162).

In the central nervous system, ATP-sensitive potassium channels (K^+_{ATP} channels) maintain resting membrane potential at more negative levels (8), mediate glucose-sensing (262), regulate neurotransmitter release (9, 13, 14), and provide neuroprotection against seizure activity (174). Autoradiography studies (167-170), *in situ* hybridization (7, 10, 171, 172), and electrophysiological techniques (173) have identified K^+_{ATP} channels in a variety of neuronal populations. The K^+_{ATP} channel is a heterodimer of four inwardly rectifying subunits (Kir6.x) and four high-affinity sulfonylurea receptor subunits (SUR). In neuronal tissue, functional channels are predominantly composed of the Kir6.2 and SUR1 subunit isoforms (7). Closure of K^+_{ATP} channels decreases membrane permeability to K^+ and shifts the membrane potential to more depolarized levels. Depolarization of the membrane induces the opening of voltage-gated Ca^{++} channels and allows Ca^{++} to enter the cell, which subsequently causes calcium-dependent

exocytosis and secretion. Conversely, opening of K^+_{ATP} channels results in increased permeability, hyperpolarization, decreased cell excitability, and therefore reduced secretion (7). Binding of ATP or channel modulators to the K^+_{ATP} channel inhibits its activity. For example, the pharmacological agent tolbutamide specifically binds to the sulfonylurea binding site on the SUR1 subunit and inhibits channel activity (263). Similar to closure of K^+_{ATP} channels, a decrease in expression of functional channels is believed to shift the membrane potential to more depolarized levels and increase cell excitability, whereas an increase in expression hyperpolarizes the cell and dampens cell excitability. Supporting this idea, it has been observed that overexpression of Kir6.2 in transgenic mice protects from hypoxic-ischemic injury (264), while mice transgenically overexpressing SUR1 in the forebrain resist seizure induction and excitotoxic neuron death (177).

Currently, the role of these channels as targets of T action and in behavior is not well defined. Using *in situ* hybridization and immunocytochemistry, Dunn-Meynell and colleagues observed that cells containing Kir6.2 mRNA were abundant in brain regions including the medial preoptic nucleus, antero-medial hypothalamus, medial amygdala, and lateral septum (10), which also express steroid hormone receptors and are involved in sexual behavior. In sexually experienced, testes-intact male rats, systemic administration of the K^+_{ATP} channel blocker glibenclamide increased male sexual behavior, while the K^+_{ATP} channel opener pinacidil reduced male sexual behavior (178). I hypothesize that T actions on sexual behavior may be mediated in part by preoptic and/or hypothalamic K^+_{ATP} channels. To address this hypothesis I assessed T's effects on expression of K^+_{ATP} channel subunit mRNA expression and the effects of pharmacological inhibition of channels on male sexual behavior in the presence or absence of testicular hormones.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Portage, WI), weighing 180-200 g at the beginning of the experiment, were individually caged, maintained on a reversed 14:10 light-dark cycle (lights off at 1000h), and given access to food and water *ad libitum*. Females of the same strain were group caged under the same conditions. All animal procedures were conducted in accordance with protocols approved by the Animal Care and Use Committee at Northwestern University.

Experiment 1: Effect of T on K^+_{ATP} channel expression

Under isoflurane anesthesia, adult male rats were sham-operated, castrated, or castrated and implanted with one or two Silastic capsules (Dow Corning, Midland, MI; 1.98 i.d., 3.18 o.d.) packed with crystalline T (30 mm length). After one week, animals were anesthetized with isoflurane and euthanized by decapitation. Trunk blood was collected and serum stored at -20°C until radioimmunoassay. For RT-PCR, the POA and MBH were rapidly dissected from the brain, frozen on dry ice and stored at -80°C until RNA extraction.

Total RNA extraction, DNase treatment, and reverse transcription were carried out as previously described (11). The cDNA product was used for PCR amplification of either Kir6.2 or SUR1 subunit and the endogenous control RPL19 to correct for total nucleic acid content. Primer sequences were: Kir6.2, sense 5′-GCTGCATCTTCATGAAAACG-3′, antisense 5′-TTGGAGTCGATGACGTGGTA-3′, 298 bp, accession no. AB043638; SUR1, sense 5′-TGGGGAACGGGGCATCAACT-3′, antisense 5′-GGCTCTGGGGCTTTTCTC-3′, 388 bp, accession no. L40624; RPL19, sense 5′-CTGAAGGTCAAAGGGAATGTG-3′, antisense 5′-

GGACAGAGTCTTGATGATCTC-3′, 195 bp, accession no. XM_235216. All primers were obtains from Genosys (The Woodlands, TX). PCR was performed in a 45 μl reaction volume containing 2 mM $MgCl_2$, 1X PCR buffer, 1.25U Taq DNA polymerase (Roche, Indianapolis, IN), 300 nM of each primer, 110 μM dNTPs, and ^{32}P-dCTP (Amersham Biosciences Corp., Piscataway, NJ). Samples were first incubated at 94°C for 4.5 minutes, then cycled through denaturing at 94°C for 30 seconds, annealing at 58°C (59°C for SUR1) for 60 seconds, and extension at 72°C for 60 seconds. A final extension was carried out at 72°C for 10 minutes. Kir6.2 and RPL19 were amplified with 31 and 23 cycles, respectively. SUR1 and RPL19 were amplified with 32 and 24 cycles, respectively. PCR products were separated by polyacrylamide gel electrophoresis and images were obtained using a phosphoimager (STORM 860, Molecular Dynamics, Sunnyvale, CA). Band density was analyzed using ImageQuant software (Molecular Dynamics, Piscataway, NJ) and normalized to RPL19. As a negative control, samples lacking reverse transcriptase during cDNA synthesis did not yield any products. RT-PCR data were analyzed with one-way ANOVA and Newman-Keuls post hoc tests.

Serum T levels were measured using a RIA kit from MP Biomedicals (Orangeburg, NY); the sensitivity and intraassay and interassay CVs were 0.02 ng/ml, 9.21%, and 7.56%, respectively. One T-filled capsule in castrated males generated serum T concentrations equivalent to those observed in intact animals. Although two T-filled capsules produced much higher T levels, concentrations remained within the high end of the physiological range (Intact, 2.17 ± 0.75 ng/ml, n=5; Cx, all at or below the level of detection, 0.02 ng/ml, n=6; Cx + low T (one T capsule), 3.71 ± 0.93 ng/ml, n=6; Cx + high T (two T capsules), 9.16 ± 3.12 ng/ml, n=6).

Experiment 2: Effect of i.c.v. tolbutamide infusion on male sexual behavior

Adult male rats were given sexual experience in a screened test for copulatory ability in which a proven sexually receptive female was placed in the home cage of the subject for 60 min. Only males achieving ejaculation were considered experienced and subsequently used in the study. The stimulus females used in the experiment were ovariectomized under isoflurane anesthesia at least one week prior to behavior testing and given injections of estrogen and progesterone to ensure maximum sexual receptivity. Estradiol benzoate (20 μg) was injected 48 h and 24 h prior to testing; progesterone (500 μg) was injected 4-7 hours before testing. Females were screened with non-experimental, sexually experienced males and only those that exhibit good sexual receptivity (i.e., solicitation behavior and lordosis response to mounting) and no rejection behavior were used.

Five weeks before behavior testing, sexually active males were sham-operated or castrated under isoflurane anesthesia. One week before testing, males were deeply anesthetized with ketamine (75 mg/kg) plus xylazine (5 mg/kg) i.p. and implanted with one 15 mm, 23-gauge thin-wall stainless steel guide cannula fitted with an obdurator (Plastics One, Roanoke, VA), stereotaxically directed to the left lateral ventricle (mm from bregma: AP, -0.5; ML, +1.5; DV, -4.5; incisor bar, 0) (265).

Ten minutes before the onset of testing, the obdurator was replaced by an infusion cannula, through which animals received a unilateral infusion into the lateral ventricle of either vehicle, 270 ng or 540 ng of freshly prepared tolbutamide (a K^{+}_{ATP} channel inhibitor) in a volume of 5 μl in 60 s using the CMA100 microinjection pump (Carnegie Medicin AB, Stockholm, Sweden). Tolbutamide (Sigma, St. Louis, MO) was first diluted in DMSO and then diluted to either 54 ng/μl (200μM) or 108 ng/μl (400μM) with artificial cerebrospinal fluid

(aCSF); its vehicle is 0.1% DMSO in aCSF. Tolbutamide is a specific inhibitor of the SUR1 subunit (266) and previous studies from my laboratory have demonstrated that these doses have significant physiological effects (11). After infusion, the infusion cannula was left in place for one minute.

All sexual behavior tests were conducted under red light illumination during the dark phase of the light cycle, beginning at least 2 hours after lights-off. Immediately following the infusion, males were given ten minutes to acclimate to the neutral Plexiglass testing arena (30 cm x 40 cm x 50 cm). Receptive stimulus females were then placed in the arena for a 30-minute test period. The following measures were recorded: mount latency, intromission latency, and ejaculation latency, mount frequency, intromission frequency, and copulatory efficacy (calculated as intromission frequency divided by mount + intromission frequencies).

To verify cannula placement, animals were infused with cresyl violet before being sacrificed. The dye traversed the ventricular system of all brains, therefore all animals were used in statistical analyses. Latency measures and frequencies of behaviors were analyzed with two-way ANOVA and Bonferroni post-hoc tests. Latencies were analyzed only for animals that performed the relevant behavior. Chi-square tests were used to analyze the percentage of each group that exhibited mounts, intromissions, and ejaculations.

Experiment 3: Effect of tolbutamide infusion into the POA on male sexual behavior

Sexually naïve adult male rats were sham-operated or castrated five weeks prior to behavior testing and implanted with one stainless steel guide cannula, stereotaxically directed to the medial preoptic area (mm from bregma: AP, +0.0; ML, +0.6; DV, -7.5) one week prior to behavior testing (265). Ten minutes before the onset of testing, all males received a unilateral

infusion into the POA of the K^+_{ATP} channel inhibitor tolbutamide (108 ng) or its vehicle in aCSF at a volume of 1 µl in 60 s. Sexual behavior tests and statistical analyses were conducted as in Experiment 2 above.

Experiment 4: Effect of i.c.v. tolbutamide infusion on locomotor activity

Following sex behavior testing in Experiment 2, intact and castrated rats were randomly reassigned to either vehicle or tolbutamide treatment groups. Rats were briefly anesthetized with isoflurane anesthesia and fitted with an acrylic tail cuff bearing a G2-eMitter transponder (MiniMitter Inc., Bend, OR), which was fixed to the tail using Nexaband Liquid Topical Tissue Adhesive (Fisher Scientific). The following day rat cages were placed on ER-4000 Energizer/Receiver plates (Minimitter Inc., Bend, OR) for two hours during the dark phase of the light cycle to monitor locomotor activity. Activity data were automatically and continuously transmitted from the transponder to the receiver plates and collected once per minute. Readings were exported to a computer running VitalView® data acquisition software (Minimitter Inc., Bend, OR). After one hour of baseline activity, rats received an infusion of vehicle or tolbutamide (540 ng) into the lateral ventricle as in Experiment 2 while freely moving in their cages. A second hour of activity was then recorded.

Results

Experiment 1: Effect of T on K^+_{ATP} channel expression

Semi-quantitative RT-PCR was used to determine the effects of T on K^+_{ATP} channel subunit mRNA expression. A one-way ANOVA revealed a significant effect of group on Kir6.2

mRNA expression in both the POA (Figure 7A) and MBH (Figure 7B) ($p<0.05$). In the POA, Kir6.2 mRNA expression was increased approximately 3.4 fold in castrates compared to sham-operated, intact controls ($p<0.05$). Castrated males receiving T replacement did not show a significant increase in Kir6.2 expression compared to intact controls. A similar trend was observed in the MBH, with Kir6.2 expression approximately 3.5 fold higher in castrates compared to intact controls ($p<0.05$). Kir6.2 expression in the MBH remained elevated in castrates receiving the low dose of T, however castrates receiving the high dose of T were not significantly different than intact controls. In contrast to the effects on Kir6.2, SUR1 mRNA expression was unaffected by castration or T replacement in the POA and MBH (Figure 7C, 7D). These results demonstrate that T exerts a suppressive effect on Kir6.2 subunit mRNA expression and are consistent with the idea that T stimulates male sexual behavior by inhibiting expression of K^+_{ATP} channels.

Experiment 2: Effect of i.c.v. tolbutamide infusion on male sexual behavior

I additionally examined the effect of pharmacological blockade of neural K^+_{ATP} channels on sexual behavior in intact and castrated male rats. Five weeks after castration, mounting behavior was still maintained in the majority of castrated male rats; however, fewer than 50% of castrates intromitted in contrast to 100% of sham-operated controls. Intracerebral ventricular (i.c.v.) infusion of the K^+_{ATP} channel inhibitor tolbutamide at the higher dose (540 ng) increased the proportion of castrated males intromitting and ejaculating compared to infusion of vehicle (Figure 8). In fact, all castrated males receiving the 540 ng dose of tolbutamide intromitted and four out of six displayed the behavioral pattern of ejaculation. A chi-square analysis revealed that significantly more tolbutamide-treated males intromitted and ejaculated than expected ($p<0.05$)

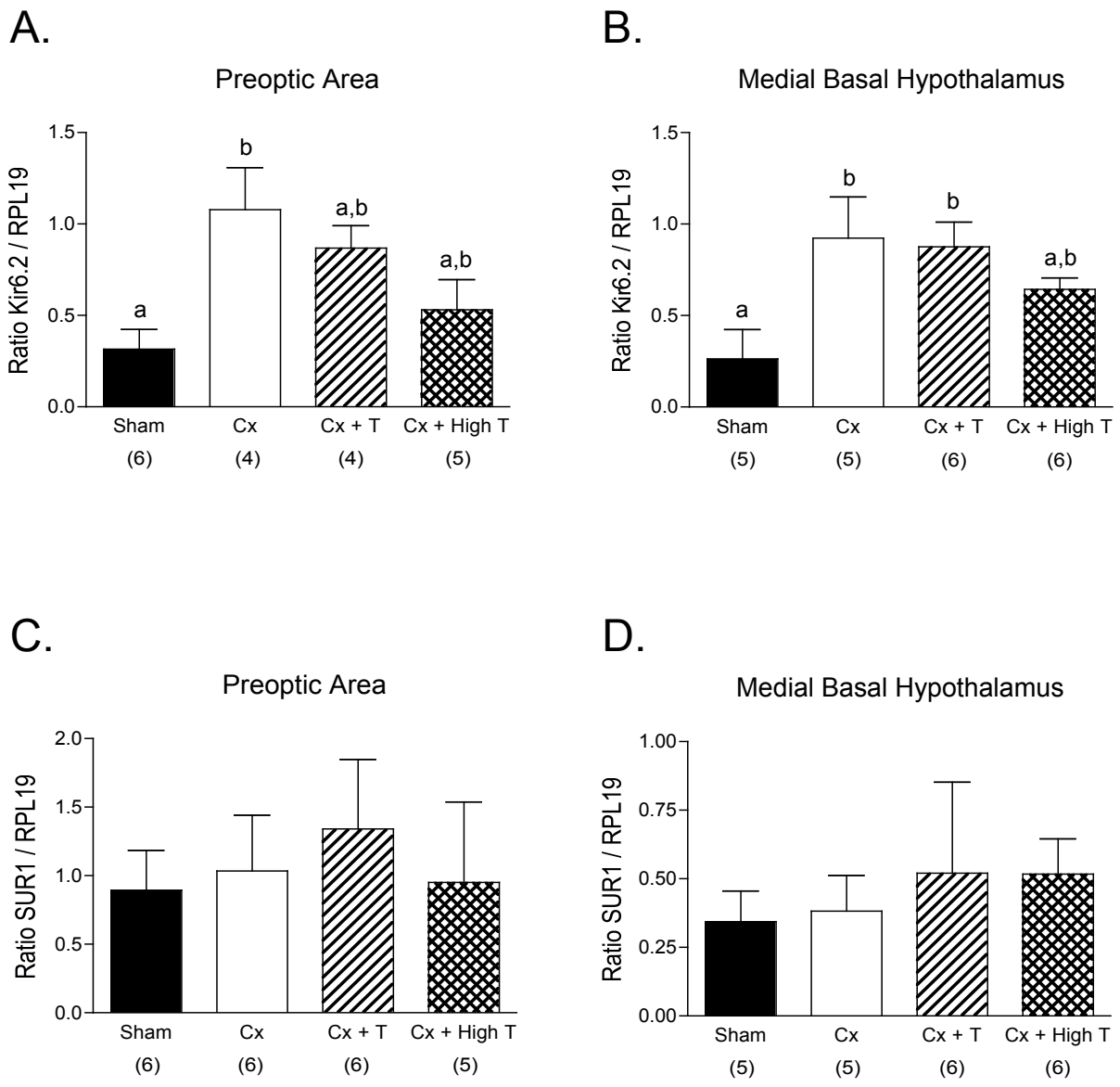


Figure 7. Effect of testosterone on mRNA expression of K^+_{ATP} channel subunits Kir6.2 and SUR1 in the preoptic area and medial basal hypothalamus. Semi-quantitative RT-PCR analysis showed that Kir6.2 mRNA expression was significantly increased in the POA (A) and MBH (B) of castrated males compared to sham-operated controls. There was no effect of treatment on SUR1 expression in either the POA (C) or MBH (D). Data are represented as mean ± SEM. Different letters indicate that groups are significantly different from one another ($p<0.05$; one-way ANOVA with Newman-Keuls post-hoc test). Sham-operated (Sham), castrated (Cx), castrated with low physiological dose of testosterone (Cx + T), castrated with high physiological dose of testosterone (Cx + High T).

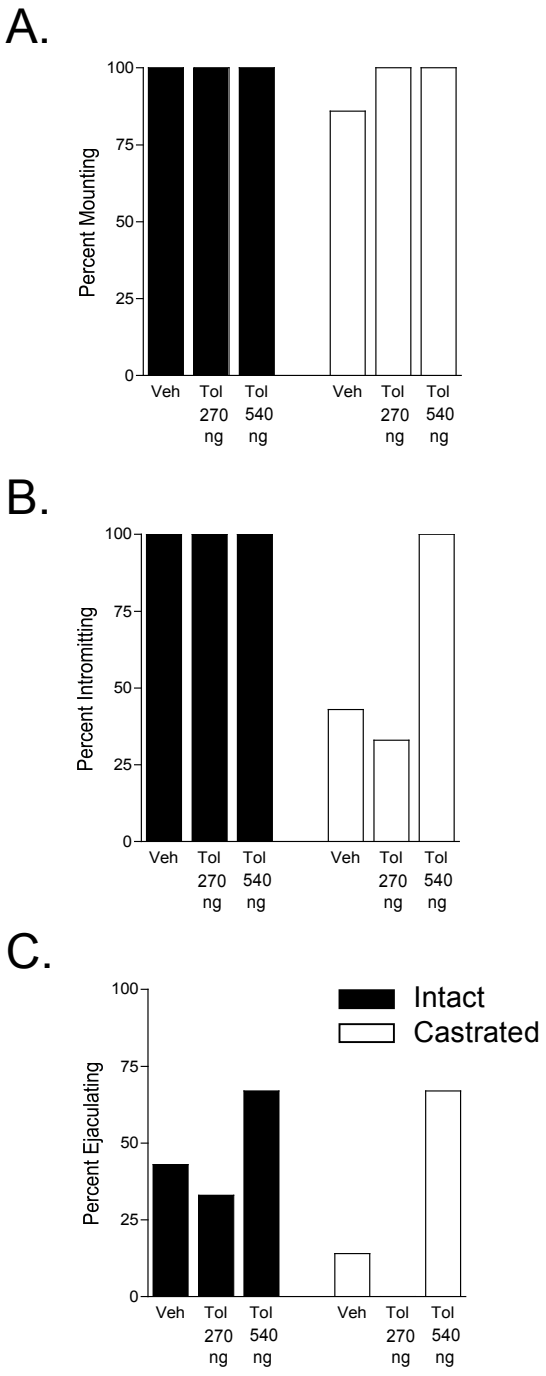


Figure 8. Central infusion of the K^+_{ATP} channel inhibitor tolbutamide restores sexual behavior in long-term castrated male rats. Adult male rats were given sexual experience, castrated (white bars) or sham-operated (black bars), and implanted with guide cannula directed to the lateral ventricle. Five weeks after castration, males were tested for sexual behavior immediately following an infusion of vehicle (Veh), or one of two doses of tolbutamide (Tol 270 ng or Tol 540 ng). Tolbutamide increased the proportion of castrated males that displayed mounts (A), intromissions (B), and ejaculation (C) compared to infusion of vehicle. ($p<0.05$, χ^2 analysis).

Latencies to mount and intromit were only calculated for those animals that displayed the behavior. A two-way ANOVA revealed a significant effect of castration ($p<0.01$), a significant effect of drug treatment ($p<0.001$), and a significant interaction between castration and drug treatment ($p<0.001$) on the latency to mount. Tolbutamide at both 270 ng and 540 ng doses significantly reduced mount latency in castrated male rats compared to infusion of vehicle ($p<0.001$; Figure 9A). These latencies were completely restored to intact levels, as they were not statistically different from those of sham-operated controls. Although statistical analyses could not be performed on intromission latencies due to the small sample sizes in the castrate group, on average, vehicle-treated castrates took approximately twice as long to intromit as castrates treated with tolbutamide (Figure 9B). Latency to ejaculate was also normal for those tolbutamide-treated castrates exhibiting the behavior ($p>0.05$ compared to Int Veh; Int Veh, 18.52 ± 6.75 min, n=3; Int Tol 270 ng, 23.57 ± 5.09 min, n=2; Int Tol 540 ng, 21.32 ± 7.09 min, n=4; Cx Veh, 29.50 min, n=1; Cx Tol 270 ng, n=0; Cx Tol 540 ng, 25.15 ± 3.55 min, n=4).

The higher dose of tolbutamide (540 ng) also significantly increased mount frequency ($p<0.05$; Figure 9C) and intromission frequency ($p<0.01$; Figure 9D) in castrate males compared to vehicle. Notably, the number of mounts and intromissions displayed by the castrates receiving 270 ng tolbutamide was not significantly different from vehicle-treated controls, indicating that the lower dose is not sufficient to facilitate these behaviors. Copulatory efficacy, a measure of intromissive success, was restored to intact levels by the higher doses of tolbutamide in the castrate group ($p<0.01$, Cx Tol 540 ng vs. Cx Veh; Int Veh, 0.80 ± 0.07, n=7; Int Tol 270 ng, 0.69 ± 0.24, n=6; Int Tol 540 ng, 0.83 ± 0.09, n=6; Cx Veh, 0.21 ± 0.37, n=4; Cx Tol 270 ng, 0.24 ± 0.37, n=6; Cx Tol 540 ng, 0.71 ± 0.23, n=6). Tolbutamide infusion did not have an effect on any of these behavioral measures in the intact group, suggesting that sexual behavior may

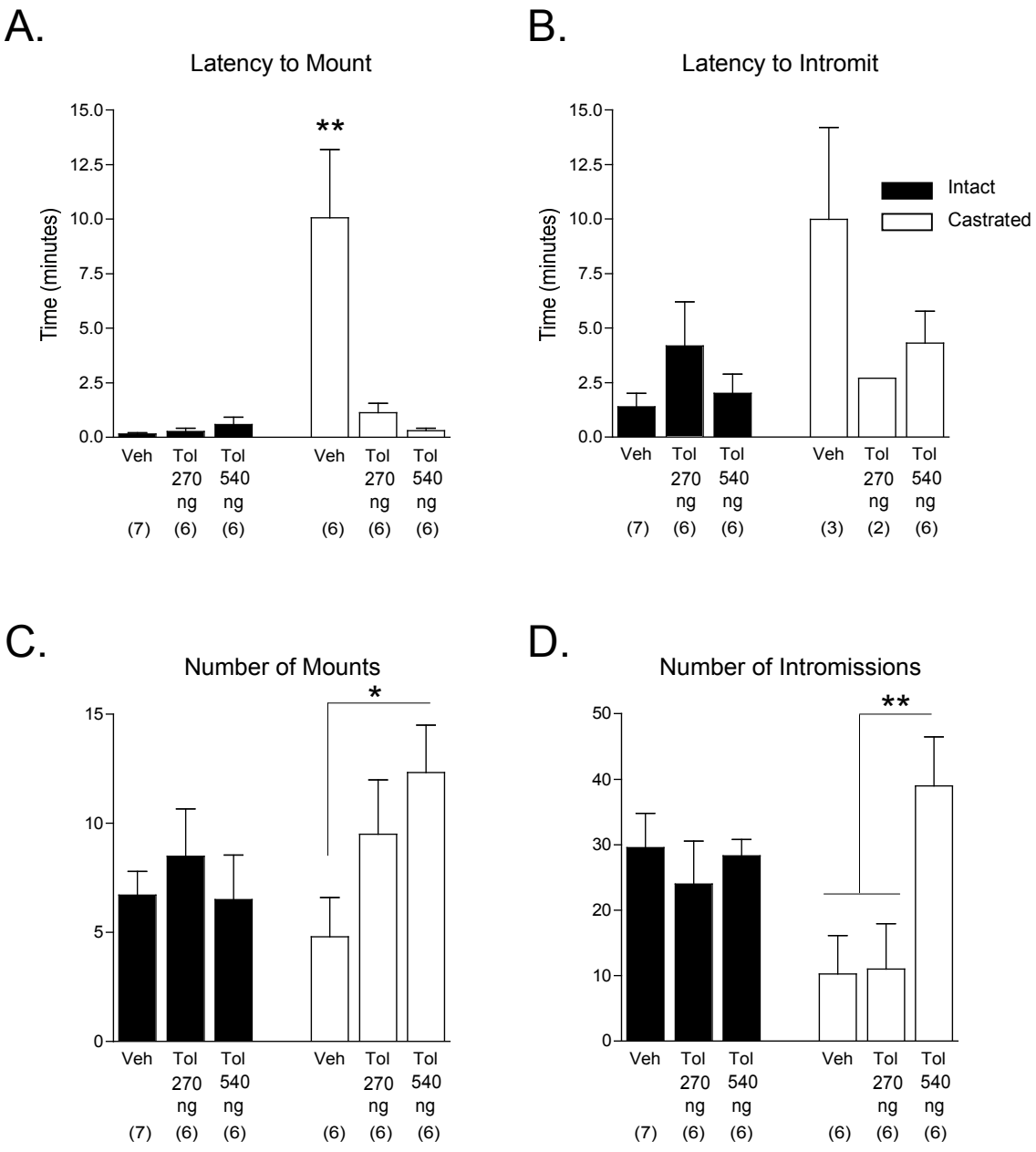


Figure 9. Mount and intromission latency and frequency. Tolbutamide infusion into the lateral ventricle significantly reduced the latency to mount (A) and reduced the latency to intromit (B) in castrated males compared to infusion of vehicle. The high dose of tolbutamide also significantly increased the number of mounts (C) and number of intromissions (D). Data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, Two-way ANOVA with Bonferroni post-hoc tests.

already be at a maximal level in this group and cannot be further stimulated by tolbutamide. Together these data demonstrate that K^+_{ATP} channel blockade is sufficient to elicit the male sexual response in the absence of T.

Experiment 3: Effect of tolbutamide infusion into the POA on male sexual behavior

As the POA is a critical integrative brain region for sexual behavior (267), I examined whether local K^+_{ATP} channel blockade in this region is sufficient to restore sexual behavior in castrates. Five weeks after castration, mounting behavior was still maintained in all castrated male rats, albeit with much greater latencies to mount; however, none displayed intromissions or ejaculation. A two-way ANOVA revealed a significant effect of castration ($p<0.01$), a significant effect of drug treatment ($p<0.05$), and a significant interaction between castration and drug treatment ($p<0.05$) on the latency to mount. Tolbutamide significantly reduced mount latency in castrated male rats compared to infusion of vehicle ($p<0.01$; Figure 10). Tolbutamide increased mount frequency in the castrated group by approximately 50% over vehicle, but this difference was not significant. In the intact group, tolbutamide had no effect on mounts, intromissions, or ejaculations. These data indicate that K^+_{ATP} channel blockade in the POA is sufficient to restore normal mounting behavior in castrated, sexually naïve rats.

Experiment 4: Effect of i.c.v. tolbutamide infusion on locomotor activity

To test whether the tolbutamide-induced recovery of sexual behavior in castrates was specific to copulation and not just a stimulation of general activity, I assessed locomotor activity following i.c.v. infusions using a radio telemetry tail clip system. Activity counts were collected for one hour before (baseline) and one hour after the infusion of vehicle or tolbutamide.

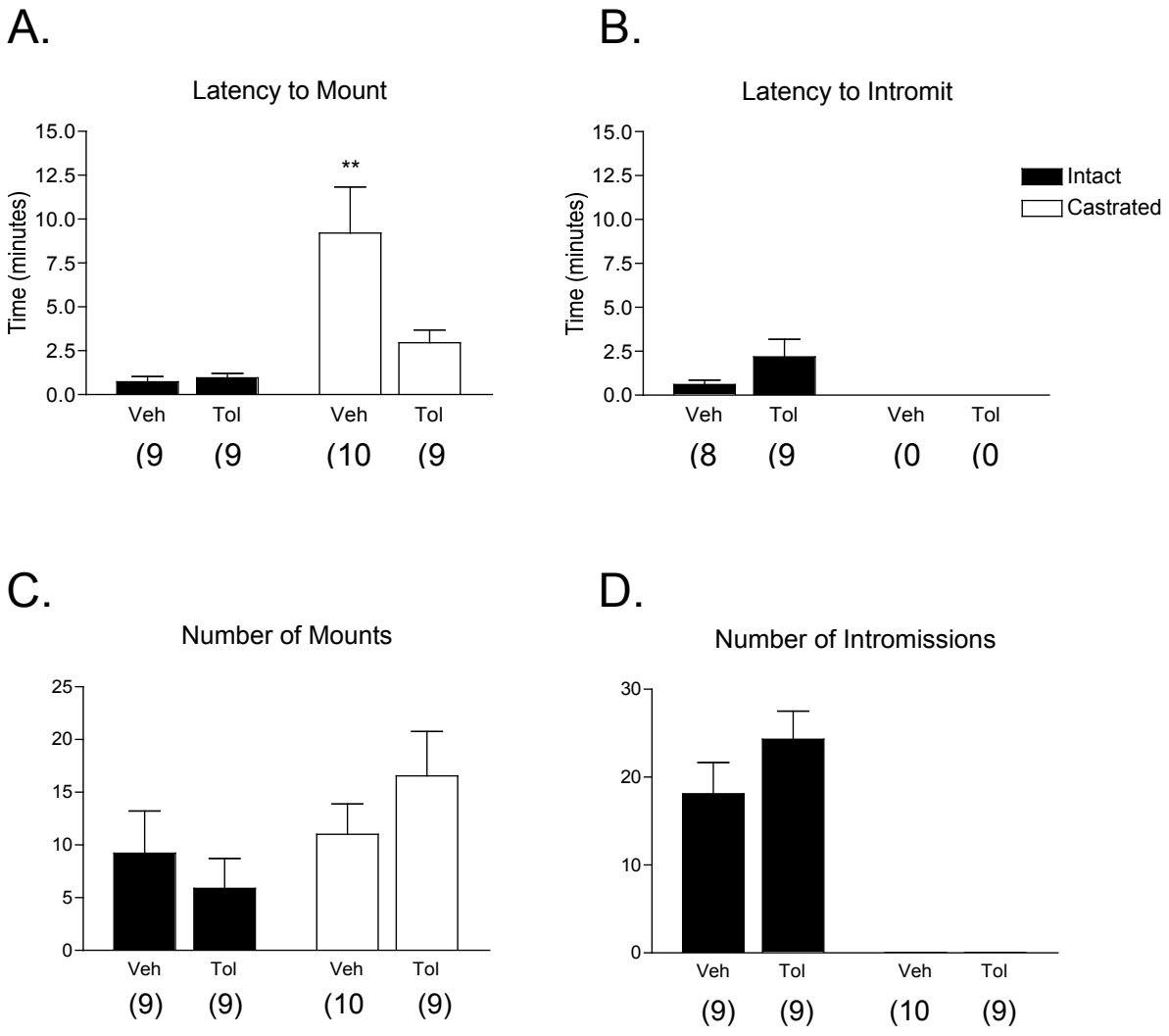


Figure 10. Preoptic area infusion of the K^+_{ATP} channel inhibitor tolbutamide restores mounting in long-term castrated male rats. Adult male rats were castrated or sham-operated, and implanted with guide cannula directed to the medial preoptic area. Five weeks after castration, males were tested for sexual behavior immediately following an infusion of vehicle (Veh) or tolbutamide (Tol; 108 ng). Tolbutamide significantly reduced the latency to mount (A) compared to infusion of vehicle. None of the castrated males displayed intromissions (B) and there was no effect of treatment on the number of mounts (C) or number of intromissions (D). Data are represented as mean ± SEM. **p<0.01, Two-way ANOVA with Bonferroni post-hoc test.

Although the vehicle-infused castrate group tended to display slightly lower baseline activity, this difference was not significant (Figure 11). Importantly, there was no effect of group on locomotor activity following the infusion, indicating that tolbutamide's effects were not due to enhancement of general locomotor activity.

Discussion

I hypothesized that ATP-sensitive potassium channels serve as a mechanism by which testosterone stimulates neuronal activity and male sexual behavior based on evidence that these channels regulate cell excitability, modulate neurotransmitter release, and are present in steroid-sensitive brain regions involved in male sexual behavior (8-10, 13, 14). In support of this hypothesis, I demonstrate here that testosterone inhibits expression of the mRNAs encoding the K^+_{ATP} channel subunit Kir6.2, and that pharmacological blockade of neural K^+_{ATP} channels can rescue sexual behavior in long-term castrated male rats. These experiments support a role for K^+_{ATP} channels in male sexual behavior and reveal a novel mechanism by which T may stimulate sexual behavior.

My analyses indicated that castration induces and T suppresses mRNA expression of the K^+_{ATP} channel subunit Kir6.2 in both the POA and MBH of adult male rats. As K^+_{ATP} channels maintain resting membrane potential at more negative levels (8), suppression of their expression by T presumably reduces membrane permeability to K^+, increases neuronal excitability and thereby renders neurons more sensitive to the hormonal, chemical, and somatosensory inputs they receive, thus increasing the likelihood of producing a behavioral response. Interestingly, SUR1 mRNA expression was unaffected by castration or T treatment. Although functional K^+_{ATP} channels have a 1:1 stoichiometry of Kir6.x and SURx subunits (175), an increase only in Kir6.2

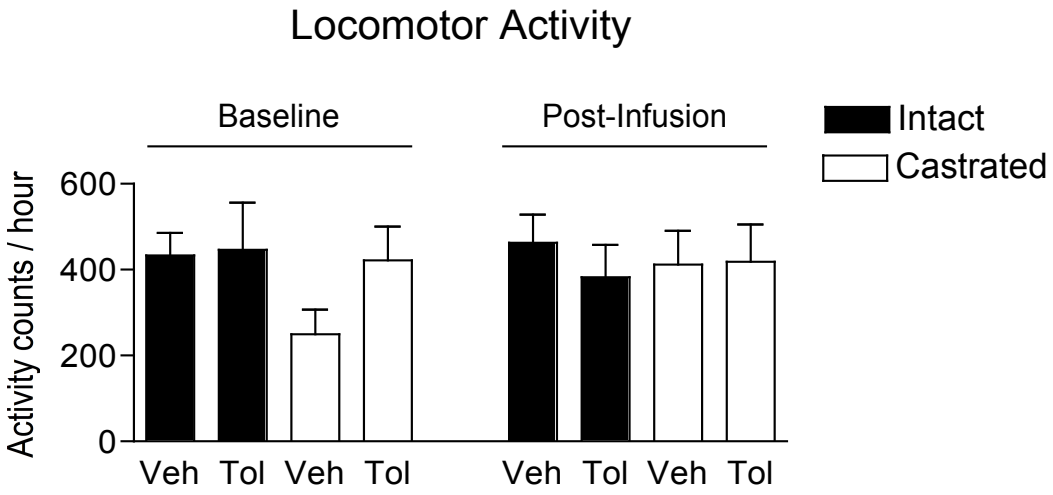


Figure 11. Infusion of the K^+_{ATP} channel inhibitor tolbutamide does not affect locomotor activity. Locomotor activity of freely moving intact and castrated rats receiving either vehicle (Veh) or tolbutamide (Tol; 540 ng) was assessed by a radiotelemetry system. Activity data were collected for one hour before the infusion (Baseline) and one hour after the infusion (Post-infusion). Data are represented as mean ± SEM. There was no effect of group on locomotor activity ($p>0.05$).

may still translate into an increase in functional channels at the membrane. For example, it has previously been shown that increased Kir6.2 expression alone in the forebrain still produces physiological K^+_{ATP} channel-dependent effects, suggesting that the pool of SURx is not limiting in the formation of the channel complex (264). Furthermore, I have recently demonstrated that stimulation of Kir6.2 expression by ovarian steroids, even in the absence of similar changes in SUR1 expression are nevertheless associated with increased neurosecretory responsiveness to tolbutamide (11). A second possibility is that the pore-forming Kir6.2 subunit may localize to the plasma membrane and function independently of the regulatory SUR1 subunit (268). Therefore, it is reasonable to suggest that, despite the lack of effect on SUR1, the castration-induced increase in Kir6.2 expression in the POA and MBH is sufficient to confer increased hyperpolarization and dampened cell excitability in these brain regions.

Regulation of K^+_{ATP} channel subunit expression has been previously demonstrated for glucose (179), glucocorticoids (12), and estrogen and progesterone (11), however, this is the first evidence to demonstrate that channel expression is regulated by testosterone in neural tissue. Although steroids can have rapid actions on sexual behavior (192-194), it is generally believed that T exerts its effects on male sexual behavior through classical transcriptional pathways. For example, recovery of sexual behavior in castrates requires several days of T treatment (132, 269), which is consistent with slower, genomic mechanisms, and treatment with a protein synthesis inhibitor blocks male sexual behavior (133). Moreover, T's effects on the electrophysiological properties of hypothalamic neurons also appear to require long-term action (4). My results demonstrating that T inhibits the expression of membrane channel subunits involved in the regulation of cell excitability are consistent with these findings. It remains to be determined whether T's suppressive effect on Kir6.2 expression is mediated via AR or via ERα

or ERβ following aromatization. It is likely that activation of both of these receptors contributes to T's effects on K^+_{ATP} channel subunit expression, as both androgenic and estrogenic metabolites are needed for the activation of male sexual behavior (270).

Infusion of the K^+_{ATP} channel inhibitor tolbutamide into the lateral ventricle increased the proportion of castrated rats engaging in copulation, increased mount and intromission frequencies, and reduced mount and intromission latencies, demonstrating that channel blockade is sufficient to restore sexual activity in castrated males to intact levels. Interestingly, few examples of such robust stimulation of copulation in castrates exist in the literature (271, 272). In contrast to the complete recovery of behavior following i.c.v. infusion, infusion of tolbutamide into the POA was only sufficient to restore mounting behavior in castrated male rats. While the POA is critical for copulation (159, 267), other brain regions make significant contributions to the full complement of sexual behavior. For example, infusions of the dopamine agonist apomorphine into the POA were only able to restore mounts to castrated males (271). Therefore, it is likely that blockade of K^+_{ATP} channels throughout the brain, as opposed to locally, is necessary to elicit the full range of copulatory behaviors.

In these studies, pharmacological blockade of neural K^+_{ATP} channels did not affect sexual behavior of intact male rats, which is in contrast to previous findings demonstrating stimulation of copulation by systemic administration of a K^+_{ATP} channel inhibitor (178). It is possible that, in the present study, sexual activity was already at maximal levels in intact males and could not be further stimulated by tolbutamide. In support of this idea, my RT-PCR data revealed low Kir6.2 mRNA expression in intact males, which would keep membrane permeability to potassium low regardless of channel activity. Alternatively, perhaps the doses of tolbutamide used here were sufficient to stimulate behavior in castrates, but higher doses would be required

to further stimulate sexual activity in intact rats. It has been previously demonstrated that an increase in Kir6.2 expression results in an increased response to channel modulators (273). Thus, the robust effect of tolbutamide in the castrated group may reflect the greater sensitivity conferred by the up-regulated expression of Kir6.2.

Suppression of K^+_{ATP} channel subunit expression by T in intact males would presumably increase excitability of androgen-target neurons and the probability of synaptic neurotransmitter release. It is well established that the neurotransmitter dopamine is important in facilitating genital reflexes, motor patterns of copulation, and sexual motivation (267, 274). Microdialysis experiments have demonstrated that DA is released in the POA of male rats in response to estrous females and copulation (131, 275-278). However, it has been shown that castrated rats do not demonstrate this DA release and do not copulate (275). In addition, compared to intact males, castrated males have lower basal levels of extracellular DA, higher intracellular DA levels, and higher total DA content, indicating that DA synthesis and storage are normal but its release is regulated by testosterone (279). Furthermore, systemic and intracranial administration of the DA agonist apomorphine is sufficient to partially restore sexual behavior to long-term castrated male rats (271, 280). These findings strongly suggest that T may maintain male sexual responsiveness in part by enhancing DA release in the POA, yet the specific mechanisms are not entirely understood.

K^+_{ATP} channels have been implicated in DA release in the rat nigrostriatal system. Microdialysis studies have demonstrated that intrastriatal infusion of pharmacological K^+_{ATP} channel inhibitors or activators produce dose-dependent increases or decreases in DA release, respectively (10, 13, 14, 281). As Kir6.2 mRNA is expressed in tyrosine hydroxylase immunoreactive (TH-ir) neurons in the striatum, it is likely that K^+_{ATP} channels directly mediate

dopaminergic neuron activity and DA release (10). I propose that these channels also regulate DA release in the hypothalamus and/or preoptic area, and that the high intracellular dopamine concentration in the POA of castrated males may reflect lowered dopamine release due to the up-regulation of K^+_{ATP} channels in the absence of androgen. The simplest model would be that K^+_{ATP} channels are present on dopaminergic nerve terminals and directly modulate cell excitability and DA release. I would therefore expect that double-label immunohistochemical studies would reveal co-localization of TH-ir and the K^+_{ATP} channel subunits $K_{ir}6.2$ and SUR1 in the medial preoptic area and anterior hypothalamus where dopaminergic terminals are located. I would also expect that infusions of the K^+_{ATP} channel inhibitor tolbutamide would increase excitability of dopaminergic neurons and thus FOS immunoreactivity in TH^+ neurons following these infusions. Future studies might also include microdialysis to measure DA release following infusion of tolbutamide (or a channel opener such as diazoxide), and administration of DA antagonists to block the behaviorally stimulating effects of tolbutamide. Together these studies would test the hypothesis that K^+_{ATP} channels mediate T-induced DA release in the hypothalamus and consequently elicit sexual behavior.

Testosterone regulation of K^+_{ATP} channel subunit expression may also have adaptive significance in the development of sexual behavior at puberty. Like in castrated adult males, low T levels in prepubertal males may keep Kir6.2 mRNA expression high in order to confer reduced excitability of steroid-sensitive neurons in brain regions involved in sexual behavior. As K^+_{ATP} channels clearly play a role in neuroprotection (282), this may effectively preserve functioning of neurons that are critical for procreation and survival of the species. For example, overexpression of Kir6.2 or SUR1 in the forebrain of transgenic mice protects from hypoxic-ischemic injury and excitotoxic neuron death, respectively (12, 264). Whether channel expression follows a temporal

pattern consistent with the pubertal rise in serum testosterone, or whether tolbutamide treatment can stimulate sexual activity in juvenile males as it did in castrates in the present study remains to be determined.

Hypothalamic K^+_{ATP} channels are critical in energy homeostasis, coupling metabolic signals to cell excitability (7). Since reproduction can be influenced by nutritional status (283), K^+_{ATP} channels may represent a common pathway by which T and metabolic signals modulate male sexual behavior. For example, ATP generated from glucose metabolism would block a greater proportion of neuronal K^+_{ATP} channels in states of positive energy balance, increasing excitability of steroid-sensitive target neurons and promoting male sexual behavior. Conversely, in states of negative energy balance, low ATP concentration would result in fewer blocked channels, dampening cell excitability and sexual behavior. Although sexual activity is generally robust in male rodents despite changes in nutritional status (284-286), evidence suggests that sexual dysfunction is associated with metabolic diseases, such as diabetes, in which there is reduced glucose utilization (287, 288). For example, sexual behavior is severely disrupted as a result of spontaneous or experimentally-induced diabetes in male rats (289-294) and some defects may be due, at least in part, to a central neuropathy (291). Furthermore, glucoprivation induced by peripheral 2-deoxyglucose (2-DG) injections reduces the number of sexual contacts made by male rats (295). Of note, 2-DG is also known to open K^+_{ATP} channels (296) and reduce neurotransmitter release *in vivo* (297). Thus, steroid hormones and metabolic cues may modulate male sexual behavior by regulating expression and gating of K^+_{ATP} channels, respectively, in neuronal circuits involved in regulating reproductive behavior.

In summary, I have identified the K^+_{ATP} channel as a potential mechanism by which testosterone and its metabolites increase neuronal activity and elicit male sexual behavior. I

determined that testosterone inhibits K^+_{ATP} channel subunit expression and that K^+_{ATP} channel blockade is sufficient to restore male sexual behavior in castrates. These experiments contribute to our understanding of the cellular mechanisms that underlie T's effects on male sexual behavior and may ultimately provide insight into potential strategies to treat human sexual dysfunction.

Importantly, the effects of T in the brain are exerted largely through conversion to E_2 and are mediated by ERα. The following chapter will explore another mechanism underlying steroid regulation of sexual behavior, namely the role of classical and non-classical ERα signaling.

CHAPTER III: ERE-INDEPENDENT ERα SIGNALING IS NOT SUFFICIENT TO MEDIATE ESTROGEN REGULATION OF MALE SEXUAL BEHAVIOR

Abstract

Estrogen receptor alpha (ERα) mediates estradiol (E_2) actions in the male brain and is critical for normal male reproductive function. In the classical pathway, ERα binds to estrogen response elements (EREs) to regulate gene transcription. ERα can also regulate gene transcription independently of EREs via protein-protein interactions with transcription factors, and additionally signal via rapid, nongenomic pathways originating at the cell membrane. This study assessed the degree to which ERE-independent ERα signaling can rescue the disrupted masculine sexual behaviors that have been shown to result from ERα gene deletion. I utilized male ERα null mice that possess an ER knock-in mutation (E207A/G208A; "AA"), in which the mutant ERα is incapable of binding to DNA and can only signal through ERE-independent pathways ($ERα^{-/AA}$ mice). I found that sexual behavior, including mounting, is virtually absent in $ERα^{-/-}$ and $ERα^{-/AA}$ males, indicating that ERE-independent signaling is insufficient to maintain any degree of normal sexual behavior in the absence of ERE binding. These data indicate that binding of ERs to EREs mediates most if not all of E_2's effects on male sexual behavior.

Introduction

Male fertility is dependent on the sex steroid hormones testosterone and 17β-estradiol. Testicular testosterone (T) secreted during pre- and perinatal periods is necessary to masculinize the external genitalia and brain. Although there is evidence for the requirement of the androgen receptor (AR) in the development and function of the male reproductive tract and masculine behaviors (15), T's effects are exerted largely through its conversion to estradiol (E_2) by aromatase and subsequent signaling through the estrogen receptor (ER) (136, 270). The importance of E_2 action in male fertility is demonstrated by descriptions of testicular dysfunction and behavioral deficits in estrogen receptor alpha knockout (ERαKO) mice. Although prenatal development of the reproductive tract does not depend on ER, ERαKO males are infertile due to atrophy of the testes and seminiferous tubules, tubule dysmorphogenesis, reduced sperm counts, and impaired copulation and other sexually motivated behaviors (16, 17, 19, 185). In contrast to ERα, deletion of ERβ does not impair testicular function, spermatogenesis, or normal masculine sexual behavior in adult mice (20-23); however, ERβ may influence the timing of puberty (22) and appears to play a role in behavioral defeminization (23, 298). This would suggest a greater requirement for ERα in the development of normal male fertility; however, it remains a possibility that ERα and ERβ regulate reproductive function together, as ERα and ERβ often form heterodimers and interactions between them have been documented in several tissues (299-304).

In the classical pathway of estrogen action, E_2 binds to the ligand-binding domain of ER, inducing conformational changes that allow the receptor to interact with coactivator or corepressor molecules. The ligand-receptor complex ultimately binds as a dimer to estrogen response elements (EREs) in the promoter region of target genes to either activate or repress

gene expression (305-308). While E_2 acts predominantly via this pathway, other mechanisms of E_2 action have also been described, such as rapid, nongenomic effects through a membrane-associated ER (96-98, 309, 310). Emerging evidence supports the existence of another pathway in which ER can regulate genes that lack an ERE via protein-protein interaction with other transcription factors, such as c-Fos/c-Jun B (AP-1), Sp1, and NF-κB (26, 81, 83, 90, 92, 93, 311-314). Whether E_2 stimulates sexual behavior via ERE-independent pathways such as these remains to be determined.

The generation of $ER\alpha^{-/AA}$ mutant mice by Jakacka and colleagues has provided a unique opportunity to distinguish between ERE-dependent and ERE-independent mechanisms of E_2 action *in vivo* (26). These mice have a mutation (E207A/G208A; "AA") in the DNA recognition sequence of ERα, which selectively eliminates ERα signaling through ERE binding and activation of ERE-containing reporter genes. This mutant receptor can signal normally through protein-protein interactions, as demonstrated by active ER regulation of reporter genes containing activator protein 1 (AP-1) response elements and ER interaction with Jun *in vitro* (26, 80). Although heterozygote $ER\alpha^{+/AA}$ males are fertile, heterozygote females display ovarian, uterine, and mammary gland defects, and are consequently infertile (26).

The goal of the present study was to examine the relative role of ERE-dependent and ERE-independent ERα actions in masculine sexual behavior. I utilized complete ERα null ($ER\alpha^{-/-}$) animals and compound heterozygotes ($ER\alpha^{-/AA}$), which lack ERE-dependent ERα signaling on both alleles and thus E_2 action can only occur through ERE-independent mechanisms. Previous studies have demonstrated that the mutant ERα allele in $ER\alpha^{-/AA}$ mice can at least partially rescue some of the phenotypes that result from ERα deletion, including elevated trabecular bone mineral density (124), loss of negative feedback on gonadotropin secretion in the female (125),

and tubular dysmorphogenesis and spermatogenesis in the testis (Weiss et. al., unpublished observations). In the present studies, I assessed the degree to which the knock-in of this mutated allele, and thus the introduction of ERE-independent ERα signaling, could similarly rescue the impaired masculine sexual behavior observed in $ER\alpha^{-/-}$ males.

Materials and Methods

Animals

All animal procedures were conducted in accordance with protocols approved by Northwestern University's Animal Care and Use Committee. The ERα null ($ER\alpha^{-/-}$) and $ER\alpha^{+/AA}$ mutant mice were generated as previously described (26, 80, 119). Breeders were backcrossed for 8 to 13 generations onto the C57Bl/6 line. Compound heterozygotes ($ER\alpha^{-/AA}$) were generated by mating heterozygote $ER\alpha^{+/AA}$ males with heterozygote ERα null females ($ER\alpha^{+/-}$). $ER\alpha^{-/-}$ mice were generated by mating $ER\alpha^{+/-}$ males and $ER\alpha^{+/-}$ females. All mice were genotyped at weaning. DNA was isolated by digestion of tail tissue and amplified in two separate PCR reactions to determine the presence or absence of the wild-type ERα and the presence or absence of the knock-in mutation.

Adult male mice were individually caged at weaning and housed under a 12:12 reversed light-dark cycle (lights off at 1000 h) with food and water available *ad libitum*. All males remained individually caged throughout the extent of the study and were not tested for behavior until they reached sexual maturity.

Stimulus female mice were group-caged, ovariectomized under isoflurane anesthesia and given s.c. injections of estradiol benzoate and progesterone to ensure maximum sexual

receptivity. Estradiol benzoate (10 µg) was injected 48 h and 24 h before testing; progesterone (500 µg) was injected 3-5 hours before testing.

Sexual behavior testing

In a protocol modified from Ogawa *et al.*, intact male mice were tested twice for masculine sexual behavior (naïve and experienced), a minimum of three days apart (180). All tests were conducted under dim red light illumination during the dark phase of the light cycle, beginning 2 hours after lights-off. Stimulus females were first screened for sexual receptivity with non-experimental, "stud" males before being placed in the experimental male's home cage for 30 minutes. Mounts and intromissions were scored according to the descriptions of McGill (183, 184). Repeated, rapid and shallow pelvic thrusting motions were scored as mounts, whereas deeper thrusts that occurred with a slower rate of pelvic thrusting than mounts were scored as intromissions. An observer blind to genotype recorded the following measures: number of mounts, number of intromissions, mount latency, intromission latency, and ejaculation latency. Sexual motivation, as indicated by time spent engaging in anogenital sniffing of the stimulus female, was also scored in a subset of animals. Following the completion of the two 30 minute tests, some animals (n=12) were tested in three additional sessions to determine if repeated sexual experience had an effect on behavior.

Statistics

The proportion of animals in each experimental group that exhibited mounts, intromissions, and ejaculation was analyzed using chi-square tests. Mount and intromission frequencies for each 30-minute test (naïve and experienced) and duration of anogenital sniffing

were initially analyzed with a Bartlett's test of equal variances. If variances differed significantly, the Kruskal-Wallis test and Dunn's multiple comparisons post hoc test were used. If the variances did not differ significantly, a one-way ANOVA and Newman-Keuls post hoc test were used. The behavioral frequency data represent all test subjects whether or not they engaged in copulation (e.g. a male that did not mount was assigned a score of zero for mount frequency). Behavioral latencies, on the other hand, were calculated only for those males that displayed the behavior. Consequently, the sample sizes for $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males were too small to perform statistical analyses on behavioral latencies.

Results

The proportion of animals exhibiting masculine sexual behavior was significantly different between genotypes in both tests (Figure 12). Almost all wild-type ($ER\alpha^{+/+}$) males mounted in both the first (94%) and second (100%) tests, most intromitted (Test 1, 71%; Test 2, 82%), and some ejaculated (Test 1, 6%; Test 2, 18%). In contrast, only one of fourteen $ER\alpha^{-/-}$ males (7%) displayed any sexual behavior; this male mounted three times over the course of two tests and achieved a single intromission. Similarly, only three of seventeen $ER\alpha^{-/AA}$ males mounted (Test 1, 18%; Test 2, 12%); two of these males intromitted (Test 1, 12%; Test 2, 6%) and none ejaculated.

In both behavioral tests, mount frequency was significantly lower in $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males compared to $ER\alpha^{+/+}$ male counterparts ($p<0.001$), as was intromission frequency ($p<0.01$, Figure 13). Wild-type males demonstrated almost twice as many mounts in the second behavioral test as in the first test, while $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males did not appear to be affected by sexual experience. To determine if this was due to a delay in their response to experience, mice

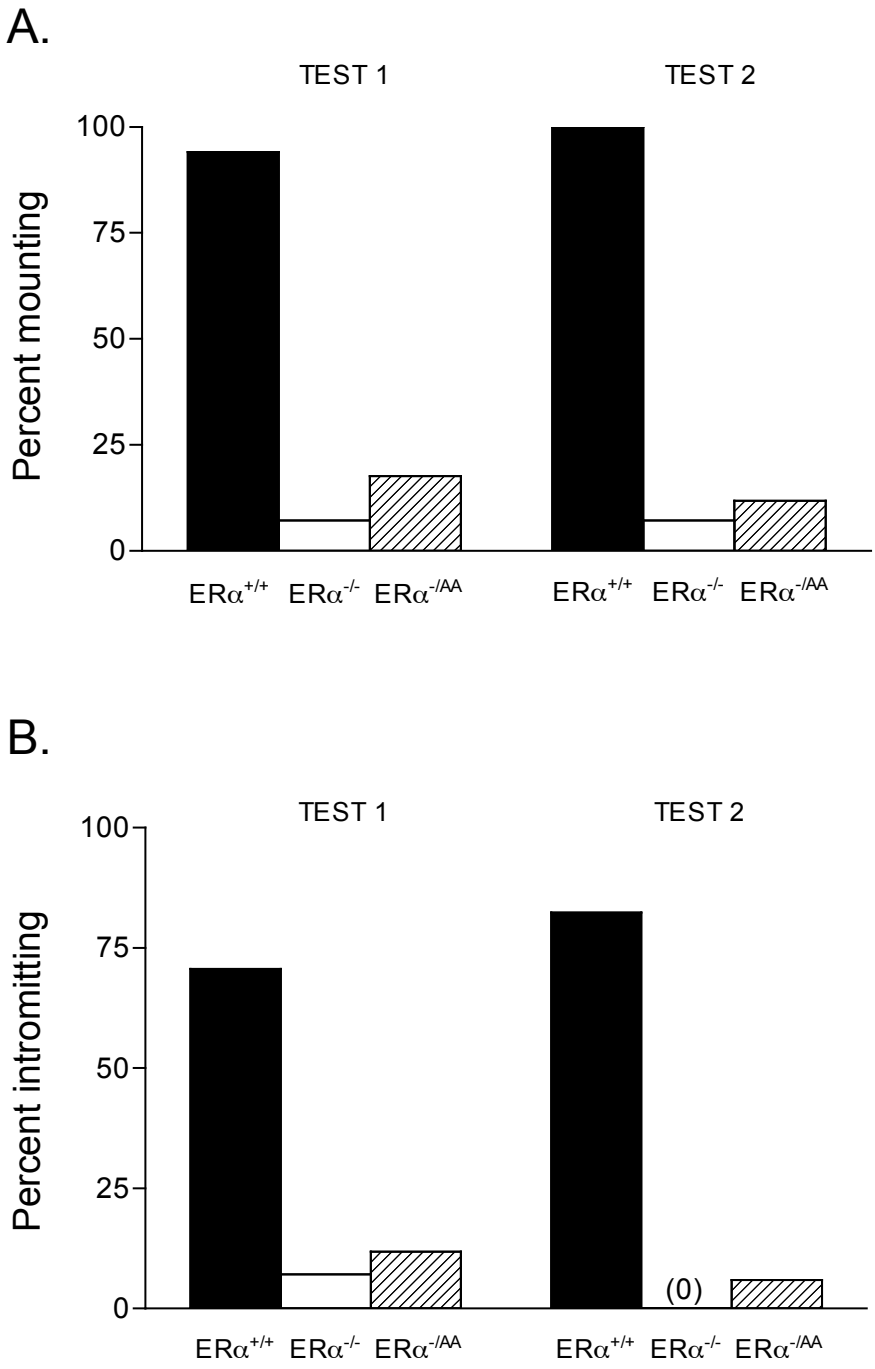


Figure 12. Proportion of male mice displaying mounts and intromissions. Wild-type ($ER\alpha^{+/+}$), estrogen receptor alpha knock-out ($ER\alpha^{-/-}$), and heterozygote mutant $ER\alpha^{-/AA}$ male mice were tested in two 30-minute tests with a sexually receptive stimulus female. Males were sexually naïve in the first test and sexually experienced in the second test. In both tests significantly fewer $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males mounted (A) or intromitted (B) than expected ($p<0.001$, chi-square analysis).

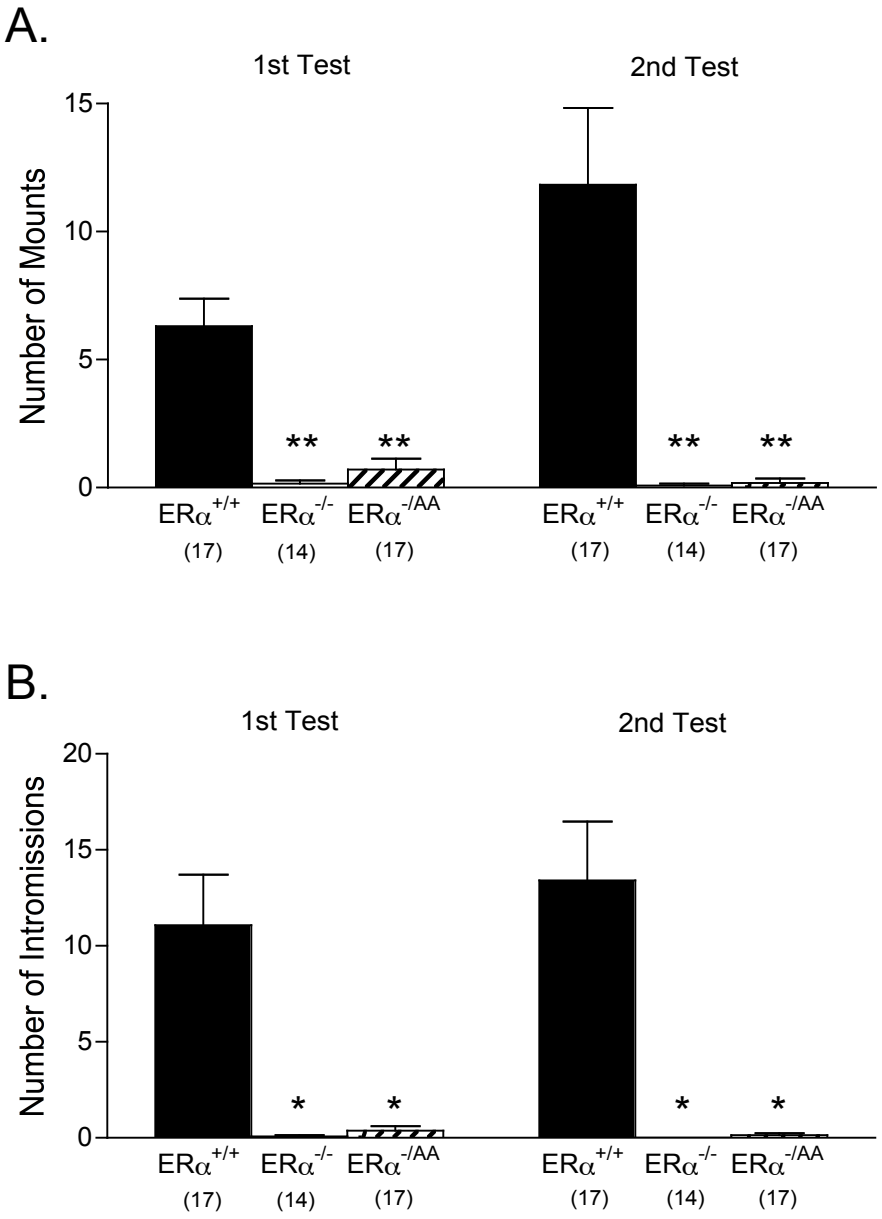


Figure 13. Mount and intromission frequency. Male mice were tested twice with a sexually receptive female; mice were sexually naïve in the first test and sexually experienced in the second test. In both tests $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males display significantly fewer mounts (A) and intromissions (B) than $ER\alpha^{+/+}$ males (*$p<0.01$, **$p<0.001$ compared to $ER\alpha^{+/+}$, Kruskal-Wallis with Dunn's multiple comparisons test). Values shown are mean ± SEM.

were tested in three additional sessions (Figure 14). ERα$^{+/+}$ males continued to display robust sexual behavior, while ERα$^{-/-}$ and ERα$^{-/AA}$ males failed to show any improvements with additional experience. In fact, both mounting and intromissive behaviors were almost completely absent in these males even in the fifth test. Sexual motivation, as indicated by sniffing the anogenital region of the stimulus female, was also impaired in ERα$^{-/-}$ and ERα$^{-/AA}$ males, indicating that the defect in their sexual behavior is not just specific to the consummatory aspects of copulation (Figure 15).

Although statistical analyses on behavioral latencies could not be performed due to the small sample sizes, the few ERα$^{-/-}$ and ERα$^{-/AA}$ males that did display mounts appear to do so with longer latencies relative to wild-types in both the first test (ERα$^{+/+}$, 12.60 ± 1.31 min, n=16; ERα$^{-/-}$, 21.82 min, n=1; ERα$^{-/AA}$, 17.55 ± 3.29 min, n=3) and second test (ERα$^{+/+}$, 9.17 ± 1.52 min, n=17; ERα$^{-/-}$, 20.95 min, n=1; ERα$^{-/AA}$, 27.06 ± 0.44 min, n=2).

Discussion

These studies assessed the role played by ERE-independent ERα signaling in male sexual behavior. My findings reveal that ERE-independent ERα signaling does not by itself maintain any aspect of masculine sexual behavior. While the majority of wild-type male mice mounted and intromitted in these tests, sexual behavior was almost completely absent in ERα$^{-/-}$ and ERα$^{-/AA}$ male mice. The few ERα$^{-/-}$ and ERα$^{-/AA}$ males that did display some sexual behavior demonstrated significantly lower behavioral frequencies than wild-type males. As there were no significant differences in behavior between ERα$^{-/AA}$ males and ERα$^{-/-}$ males, we conclude that one ERE-independent ERα mutant allele is not sufficient, and ERE-dependent ERα signaling is essential, to maintain normal male behavior.

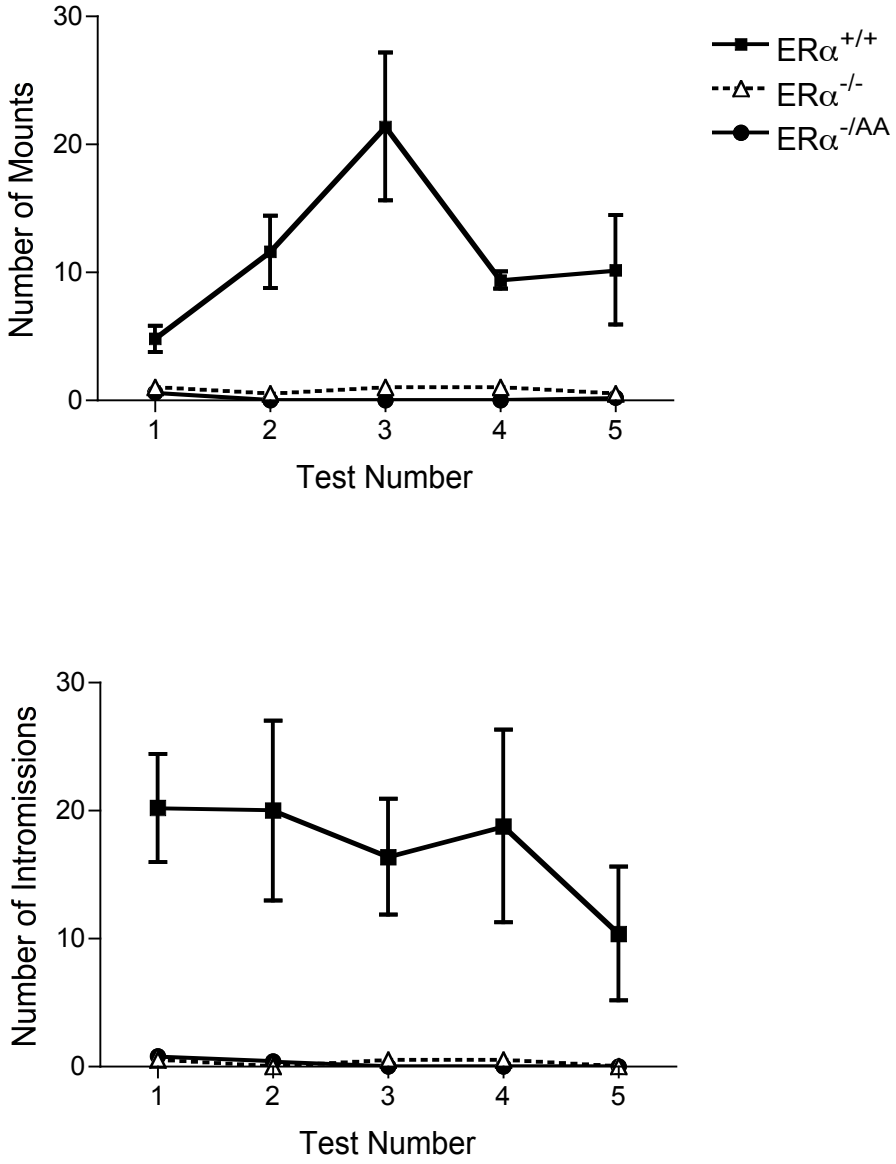


Figure 14. Mount and intromission frequency in extended tests for sexual behavior. Male mice were tested in five weekly tests with a sexually receptive female; mice were sexually naïve in the first test and sexually experienced in subsequent tests. ERα$^{-/-}$ and ERα$^{-/AA}$ males displayed fewer mounts (A) and intromissions (B) than ERα$^{+/+}$ males in all five tests. Values shown are mean ± SEM.

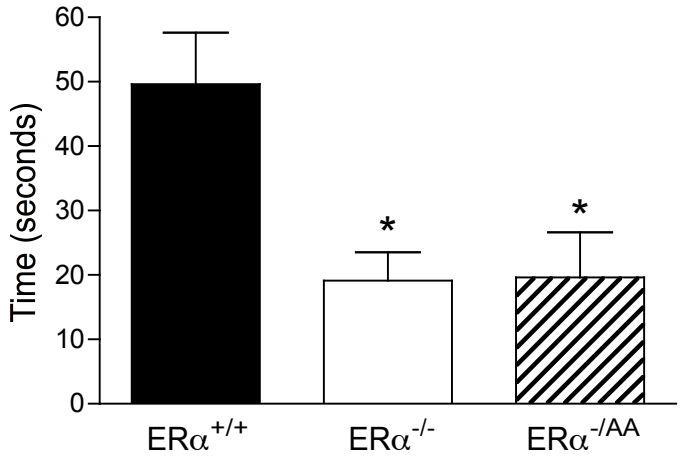


Figure 15. Duration of anogenital sniffing. ERα$^{-/-}$ and ERα$^{-/AA}$ males spend significantly less time than ERα$^{+/+}$ males engaging in anogenital sniffing of a sexually receptive female during tests for sexual behavior, indicating that sexual motivation is impaired in these groups. Data represent mean ± SEM. n=3 per group. *$p<0.05$, one-way ANOVA with Newman-Keuls post hoc test.

These results demonstrate that sexual behavior depends on ERα-DNA binding and are consistent with previous *in vivo* studies that demonstrate the importance of genomic actions of E_2 on masculine sexual behavior. Reinstating copulation after castration with steroid hormones usually takes days to weeks, which suggests that longer-term genomic effects are necessary. This is supported by findings that the protein synthesis inhibitor anisomycin blocks the effects of T (and E_2) on copulatory behavior when implanted into the male rat POA (133). The mutant ERα in the ERα$^{-/AA}$ model can alter gene transcription at non-ERE sites by tethering to other transcription factors such as Jun (80). The lack of mounts and intromissions in these animals, however, suggests that transcription through non-ERE sites is not sufficient to maintain sexual behavior. Thus, gene targets of E_2 action that contribute to the expression of behavior likely contain EREs.

There are a variety of non-ERE-mediated mechanisms that have been proposed for E_2 action. As just one example, E_2 can signal via rapid, nongenomic actions originating at the cell surface. It is not entirely clear whether a membrane-associated ERα plays a role in the central control of sexual behavior, although E_2 can have rapid actions on neuronal firing in male POA slices (191). Presumably, the mutant ERα in the ERα$^{-/AA}$ model is capable of translocating to the plasma membrane and mediating nongenomic effects of E_2, as the knock-in mutation is specific to the DNA-binding domain and leaves the membrane-localization domain intact (80). While further analyses are needed to obtain direct evidence that this is possible, the fact that ERα$^{-/AA}$ males display severely impaired behavior compared to ERα$^{+/+}$ counterparts would suggest that if there are any nongenomic actions of E_2 occurring through a membrane-associated ERα, they are not sufficient to maintain normal masculine sexual behavior in the absence of ERE-dependent pathways.

It is possible that the absence of sexual behavior in $ER\alpha^{-/AA}$ males is due to the fact that only one allele of the mutant ERα is present and is therefore insufficient to elicit significant effects. Other studies of $ER\alpha^{-/AA}$ mice support the idea that one mutant allele is enough to at least partially rescue the phenotypes generated by the deletion of the wild-type ERα. For example, $ER\alpha^{-/-}$ animals display elevated trabecular bone mineral density, whereas $ER\alpha^{-/AA}$ animals have levels similar to those of wild-types (124). In the female, the knock-in mutation restores negative feedback on gonadotropin secretion, which is absent in $ER\alpha^{-/-}$ females (125). Finally, testicular degeneration, epididymal dysfunction, and increased T secretion are all observed in $ER\alpha^{-/-}$ males but not young $ER\alpha^{-/AA}$ males (Weiss et.al., unpublished observations). However, different physiological processes can have different gene expression requirements, and two copies of the mutant ERα may be necessary for the restoration of sexual behavior. Accordingly, a role for ERE-independent ERα signaling in male sexual behavior cannot be ruled out completely. It would therefore be especially interesting to study the AA mutation in the homozygous state to determine if gene dosage does in fact have an effect on sexual behavior. Unfortunately, the apparent infertility of heterozygous females ($ER\alpha^{+/AA}$) makes generating these animals difficult (26).

It is important to note that some studies suggest that E_2 and ER signaling may not be essential for the expression of sexual behavior in adulthood. Despite evidence that neonatal E_2 may play a role in masculinization of sexual behavior, male aromatase knockouts, which cannot convert T to E_2, can sire litters when they are young adults, but demonstrate reduced fertility with age (233, 234, 315). ERαKO males display normal sexual behavior when given the dopamine agonist apomorphine, suggesting that the brain is sufficiently organized and that ERα is not necessary during development or adulthood for the expression of male sexual behavior.

Furthermore, other studies support AR-dependent masculinization. For example, gonadally intact androgen receptor knockout males exhibit no male sexual behavior. Although E_2 treatment resulted in recovery of mounts and intromissions in androgen receptor knockout males, ejaculation was not restored and any E_2-induced recovery was only about 50% of that observed in wild-type mice (15). The ability of DHT treatment to restore mounts and intromissive behaviors in castrated $ER\alpha^{-/-}$ males also suggests the importance of androgen-signaling (15). However, this study indicates that AR is not sufficient to maintain normal behavior in intact animals, as T conversion to DHT and AR stimulation can occur in $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males, yet sexual behavior was virtually absent. Similarly, the present study further demonstrates that ERβ is not sufficient for the expression of copulation in $ER\alpha^{-/-}$ males under these conditions. This is in contrast to the findings of Ogawa and colleagues, who have proposed that, because ERαβKO males do not display behavior, either one of the ERs is sufficient for the expression of simple mounting in male mice, indicating a redundancy of function (182).

In summary, I have demonstrated that ERE-independent signaling via ERα in $ER\alpha^{-/AA}$ mice is not sufficient to recover masculine sexual behavior in the absence of ERE-dependent mechanisms, indicating that signaling through EREs mediates most if not all of E_2's effects on male sexual behavior. Understanding the molecular mechanisms by which ERα mediates its effects in specific physiological systems will ultimately be helpful in the development of pharmacological therapies that differentially modulate ERE-dependent and -independent processes. The following chapter further explores the relative contributions of these ERα signaling pathways to E_2 regulation of the male reproductive axis.

CHAPTER IV: ERE-INDEPENDENT ERα SIGNALING MEDIATES ESTROGEN INHIBITION OF ANDROGEN BIOSYNTHESIS IN THE MOUSE TESTIS

Abstract

Estrogen receptor alpha (ERα) mediates estrogen actions in the male reproductive axis and is essential for normal fertility. This study assessed whether ERE-dependent and/or ERE-independent ERα signaling mechanisms contribute to estrogen regulation of gonadotropin and testosterone secretion. As in the previous chapter, I utilized $ER\alpha^{-/AA}$ male mice, in which the mutant is incapable of binding to EREs. I found that serum LH levels were not affected by genotype, demonstrating that ERα is not necessary for estrogen negative feedback in the male, and that serum FSH levels were suppressed in $ER\alpha^{-/AA}$ males but not $ER\alpha^{-/-}$ males. The knock-in mutation was sufficient to restore the elevated serum testosterone (T) levels that result from ERα gene deletion, suggesting that estrogen may specifically inhibit androgen biosynthesis via ERE-independent mechanisms. When cultured *in vitro*, testes from $ER\alpha^{-/-}$ mice secrete approximately two- to three-fold more T than wild-type counterparts, whereas testes from $ER\alpha^{-/AA}$ mice produce normal amounts of T. Real-time PCR results indicate that steady state mRNA levels for StAR, 17α-hydroxlyase ($P450_{17\alpha}$), P450 side chain cleavage (P450scc), 17β-hydroxysteroid dehydrogenase, and 3β-hydroxysteroid dehydrogenase (3β-HSD), are significantly elevated in the testes of $ER\alpha^{-/-}$ but not $ER\alpha^{-/AA}$ mice. Increased T production in $ER\alpha^{-/-}$ males is also associated with higher steroidogenic enzyme activity of 3β-HSD, P450scc, and $P450_{17\alpha}$ as measured by conversion of radiolabeled steroid substrates to T or its precursors, while enzyme activity of 3β-HSD and P450scc in $ER\alpha^{-/AA}$ testes was not different from wild-type testes. These data indicate that non-classical ERα signaling mediates estrogen's inhibitory effects on T production.

Introduction

Testosterone (T) production from testicular Leydig cells is essential for the development and maintenance of normal male reproductive function. Leydig cells also synthesize the natural estrogen 17β-estradiol and express two known estrogen receptor subtypes ERα and ERβ, and are thus sensitive to estrogen action (65, 236, 253). It is well known that endogenous and excess estrogens inhibit T production during both fetal and neonatal development (237) and in adulthood (25), however, the molecular mechanisms of their action are only recently being identified. Several studies suggest that ERα largely, if not solely, mediates estrogen inhibition of androgen biosynthesis, as serum T levels are significantly elevated in the ERαKO male mouse (16, 25, 229, 254, 255) but normal in ERβKO males (20). Furthermore, estrogen treatments effectively reduce serum T in WT males but not $ER\alpha^{-/-}$ males, suggesting that the presence of ERβ in $ER\alpha^{-/-}$ testes is not sufficient to mediate estrogenic actions on T secretion (25). Importantly, evidence suggests that the elevation of serum T due to ERα deletion is independent of changes in LH stimulation (16, 229, 255) and more likely reflects a direct enhancement of androgen biosynthesis in individual Leydig cells (25).

In the classical pathway of estrogen action, estrogens bind to estrogen receptors, inducing conformational changes within the receptor that promote dimerization and interaction with coactivator and corepressor molecules. The ligand-receptor complex binds with high affinity to specific estrogen response elements (EREs) in the regulatory regions of target genes to either activate or repress gene expression (305-308). Non-classical mechanisms of estrogen action have also been described, including rapid, non-genomic effects through a membrane-associated estrogen receptor (96-98, 309, 310), and ERE-independent genomic signaling, in which the estrogen receptor regulates genes independent of direct DNA binding via protein-protein

interaction with other transcription factors, such as Fos and Jun (AP-1), Sp1, and NF-κB (26, 81, 83, 90, 92, 93). Of note, a G-protein-coupled estrogen receptor was shown to be present in the adult testis (258), and estrogen has been shown to suppress expression of P450 steroidogenic enzyme genes indirectly via inhibition of SF1 expression (253, 259). Moreover, evidence suggests that estrogens and xenoestrogens inhibit androgen production via a non-genomic action mediated by an estrogen membrane receptor in Atlantic croaker (260). Whether estrogens specifically inhibit androgen biosynthesis via non-classical, ERE-independent ERα mechanisms in mice remains to be determined.

As demonstrated in Chapter III, non-classical estrogen receptor knock-in mice ($ER\alpha^{-/AA}$) provide a unique opportunity to distinguish between ERE-dependent and ERE-independent mechanisms of estrogen action *in vivo* (26). The goal of the present study was therefore to examine the relative role of ERE-dependent (classical) and ERE-independent (non-classical) ERα actions in T biosynthesis and gonadotropin secretion in the male. My findings demonstrate that ERE-independent mechanisms are sufficient to mediate estrogen inhibition of testicular T secretion, steroidogenic enzyme gene expression, and steroidogenic enzyme activity, independent of changes in LH.

Materials and Methods

Animals

ERα null ($ER\alpha^{-/-}$) and $ER\alpha^{+/AA}$ mutant mice were generated as previously described in Chapter III. Adult male mice were group-housed under a 12:12 light-dark cycle with food and water available *ad libitum* and were castrated at 3 to 5.5 months of age. Each testis was weighed and used for the experiments outlined below.

Hormone measurements

Intact male mice were deeply anesthetized with ketamine and xylazine i.p., and blood was withdrawn via cardiac puncture at 1500 h. Blood was centrifuged and serum stored frozen at −20°C until radioimmunoassay. Serum from each animal was assayed for LH, T, and FSH. Serum LH levels were determined using RP-3 standard and S-11 antibody, generously provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); the sensitivity and intraassay and interassay coefficients of variance (CV) were 0.01 ng/tube, 4.87%, and 8.20%, respectively. Serum FSH levels were determined using RP-2 standard and S-11 antibody, also from NIDDK; the sensitivity and intraassay and interassay CV were 0.05 ng/tube, 19%, and 14.6%, respectively. Serum T levels were measured using a RIA kit from MP Biomedicals (Orangeburg, NY); the sensitivity and intraassay and interassay CV were 0.02 ng/ml, 8.28%, and 13.9%, respectively.

In Vitro Testis Culture

Immediately following castration, one half of one testis from each animal was weighed and incubated in 1 mL DMEM/F-12 culture medium containing 0.1% BSA and 0.5 mg/ml bovine lipoprotein and buffered with 14 mM $NaHCO_3$ at 34°C with shaking for three hours. A 100 μL aliquot of media was collected at 90 minutes and replaced with fresh media containing 100 ng/ml ovine LH. A final sample was taken 90 minutes after the addition of LH. T levels in the spent media were measured by radioimmunoassay using a kit from MP Biomedicals (Orangeburg, NY); the sensitivity and intraassay and interassay CV were 0.02 ng/ml, 8.28%, and 13.9%, respectively. T concentrations were normalized to the amount of tissue cultured.

RNA Isolation and Real-time PCR

One half of one testis was frozen on dry ice and stored at -80°C. RNA was extracted using the RNeasy Mini kit (Qiagen Inc., Valencia, CA). RNA concentration was determined by measuring absorbance at 260 nm with a spectrophotometer and 1 μg total RNA was reverse transcribed into cDNA as previously described (11). Real-time PCR was conducted using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) as the primer/probe sets for the following genes: *Star* (Mm_00441558_m1), *Cyp17a1* (Mm_00484040_m1), *Cyp11a1* (Mm_00490735_m1), *Hsd17b3* (Mm_00515131_m1), *Hsd3b1* (Mm_00476184_g1), *Hsd3b6* (Mm_00834440_m1), *Srd5a1* (Mm_00614213_m1), and *Cyp19a1* (Mm_00484049_m1). Each sample was run in duplicate and carried out in a 20 μl total reaction volume per well on a 96-well plate. Each well contained TaqMan Universal PCR Master Mix reagent, the target primer/probe, endogenous control primer/probe, and 4 ng of cDNA. Expression levels for each sample were quantitated by the relative standard curve method and normalized to the internal standard 18S.

Steroidogenic Enzyme Activity Measurements

The activities of 3β-HSD, $P450_{17\alpha}$, and 17β-HSD were determined by incubation of testicular tissue with radiolabeled substrates and separation of products by thin layer chromatography as previously described (25, 316). The activity of 3β-HSD was determined by measuring conversion of pregnenolone to progesterone. $P450_{17\alpha}$ catalyzes two mixed function oxidase reactions: 17α-hydroxylation and $C_{17–20}$ cleavage. 17α-hydroxylation activity was determined by measuring the conversion of progesterone to 17α-hydroxyprogesterone, androstenedione, and T, whereas $C_{17–20}$ cleavage activity was indicated by the conversion of

17α-hydroxyprogesterone to androstenedione and T. The activity of 17β-HSD was assayed by measuring the conversion of androstenedione to T. The reaction mixtures contained 1 μM substrate (1 μCi) and were maintained at 34°C and pH 7.2 in a shaking water bath for 10 min. Ice-cold ethyl acetate was added to sample tubes to terminate reactions, then steroids were rapidly extracted and the organic layer dried under nitrogen. Steroids were separated on thin layer chromatography plates, and radioactivity was measured using a radiometric scanner (System 200/AC3000, Bioscan, Inc., Washington DC).

The activity of P450scc was determined by measuring the conversion of side-chain-labeled 25-[26,27-^{3}H] hydroxycholesterol to radioactive 4-hydroxy-4-methylpentanoic acid as previously described (25, 317). Incubations were performed for 30 min at 34°C. The reaction mixture was terminated with 0.5 M NaOH, extracted twice with chloroform, and mixed with neutral alumina to remove non-metabolized substrate. An aliquot was removed for measurement by liquid scintillation counting.

Statistics

All data were analyzed using one-way ANOVAs with Newman-Keuls post hoc tests.

Results

Serum hormone levels

Basal testosterone levels were significantly elevated in ERα$^{-/-}$ males (2.70 ± 0.77 ng/ml; n=13; Figure 16A) compared to ERα$^{+/+}$ (0.91 ± 0.36 ng/ml; n=13; $p<0.05$) and ERα$^{-/AA}$ animals (0.72 ± 0.52 ng/ml; n=9; $p<0.01$). Plasma T levels of ERα$^{-/AA}$ males were similar to those of wild-type males. This approximately 3-fold increase in T levels in ERα$^{-/-}$ males is consistent with

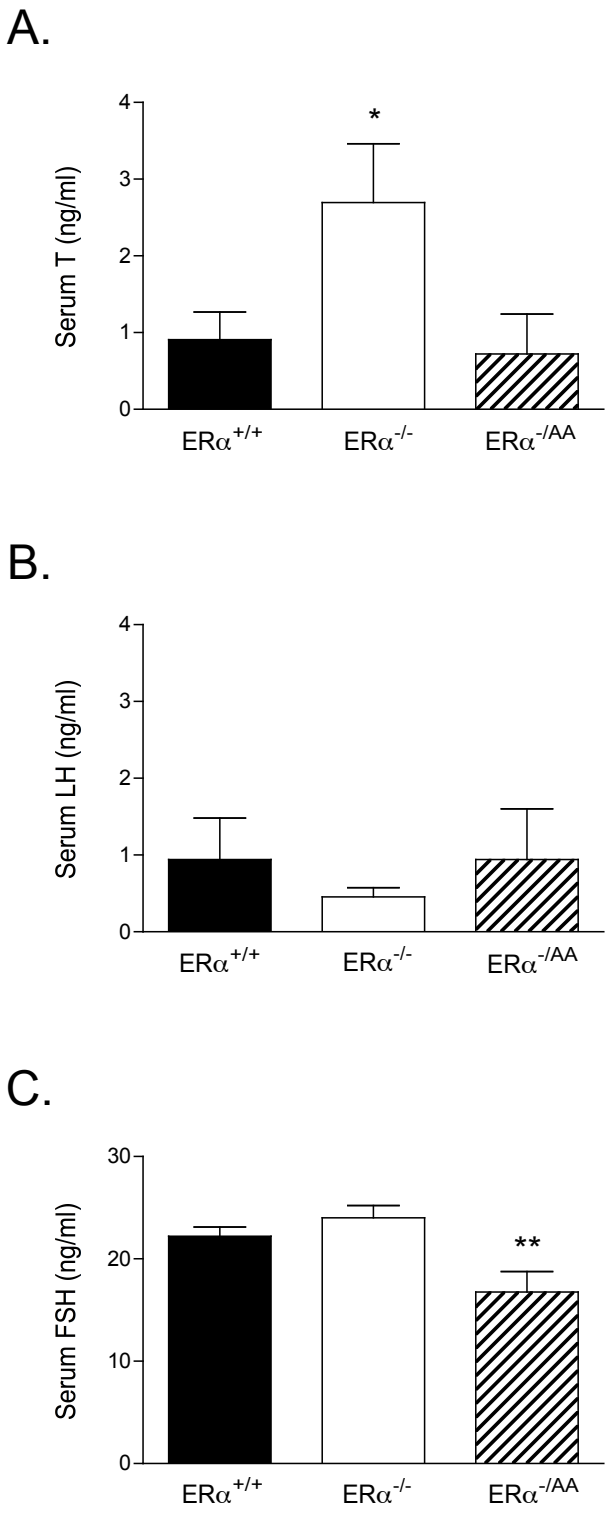


Figure 16. Serum hormone levels. (A) Serum T levels are significantly elevated in $ER\alpha^{-/-}$ males compared to $ER\alpha^{+/+}$ (*$p<0.05$) and $ER\alpha^{-/AA}$ males ($p<0.01$). (B) There is no effect of genotype on serum LH levels. (C) Serum FSH levels are significantly lower in $ER\alpha^{-/AA}$ males compared to $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ males (**$p<0.01$). Values shown are mean ± SEM. n=9-13 per group.

previous reports (16, 19). There was no effect of genotype on LH levels ($p>0.05$; $ER\alpha^{+/+}$, 0.94 ± 0.54 ng/ml, n=13; $ER\alpha^{-/-}$, 0.46 ± 0.12 ng/ml, n=13; $ER\alpha^{-/AA}$, 0.95 ± 0.66 ng/ml, n=9; Figure 16B). Basal FSH levels were significantly lower in $ER\alpha^{-/AA}$ males (16.77 ± 1.99 ng/ml; n=9) compared to $ER\alpha^{+/+}$ (22.20 ± 0.95 ng/ml, n=13) and $ER\alpha^{-/-}$ males (24.02 ± 1.19 ng/ml, n=13; Figure 16C).

Testosterone production in vitro

To determine if the knock-in mutation directly rescues T secretion by the testis, I measured T production from testes cultured *in vitro*. After 90 minutes in culture, basal T secretion from $ER\alpha^{-/-}$ testes was approximately 2.3-fold greater than that from both $ER\alpha^{+/+}$ and $ER\alpha^{-/AA}$ testes (Figure 17A). There was a significant effect of genotype on LH-stimulated T secretion ($p<0.05$), with T levels from $ER\alpha^{-/-}$ testes approximately two- to three-fold greater than that from $ER\alpha^{+/+}$ and $ER\alpha^{-/AA}$ testes, respectively (Figure 17B). As $ER\alpha^{-/AA}$ testes produced relatively normal levels of T throughout the culture period, these data demonstrate that ERE-independent ERα signaling is sufficient to mediate estrogen suppression of testicular T production.

Steady state mRNA expression of StAR and steroidogenic enzymes

In the first and rate-limiting step of androgen biosynthesis, StAR facilitates the transport of the steroid substrate cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane of Leydig cells. Subsequent conversion of cholesterol to T requires four enzymes: P450scc, $P450_{17\alpha}$, 17β-HSD, and 3β-HSD. Estrogen regulates the expression of the genes encoding these enzymes (253) and testes from adult $ER\alpha^{-/-}$ males have been reported to

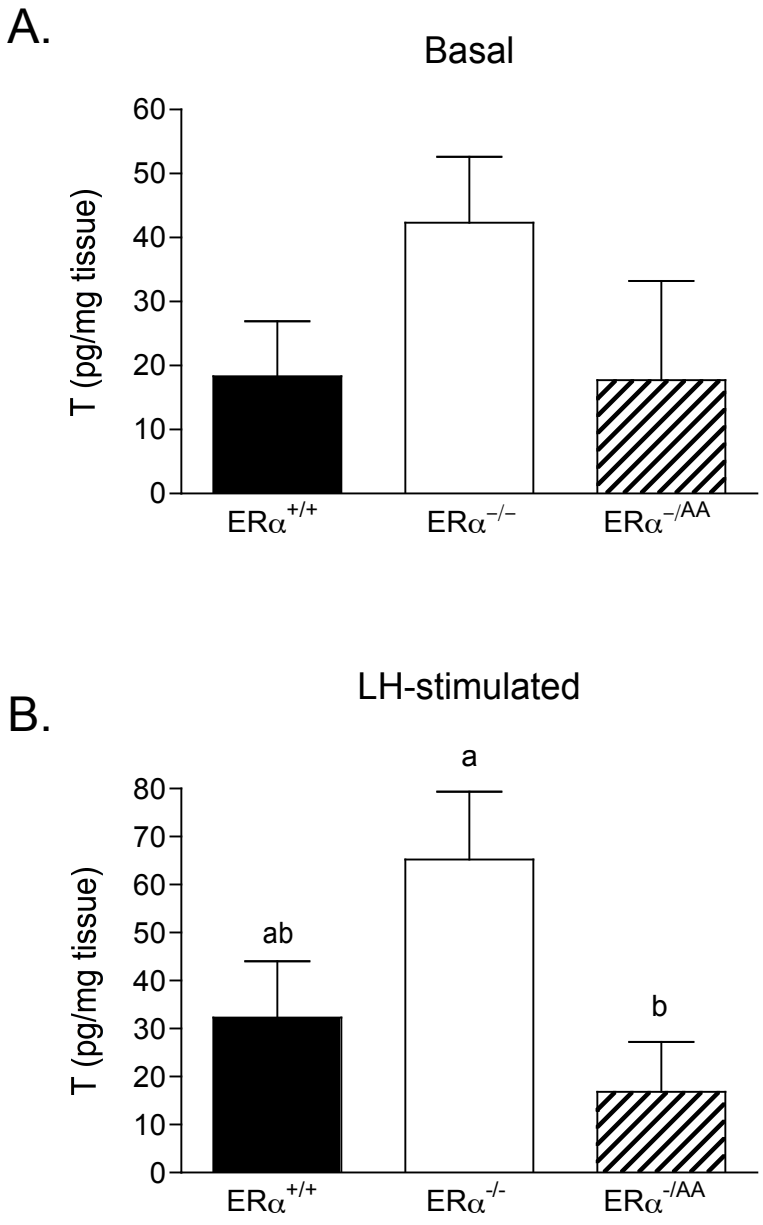


Figure 17. Testicular testosterone production *in vitro*. Basal (A) and LH-stimulated (B) T secretion is two- to three-fold higher in $ER\alpha^{-/-}$ testes compared to $ER\alpha^{+/+}$ and $ER\alpha^{-/AA}$ testes. T concentration in the spent media was measured by RIA and normalized to the amount of tissue cultured. Data represent mean ± SEM. n=4-6 per group. Different letters indicate that groups are significantly different from one another ($p<0.05$).

express higher steady state mRNA levels of StAR, $P450_{17\alpha}$, and 17β-HSD (25). Expression levels of StAR, $P450_{17\alpha}$, and P450scc mRNA have also been shown to be elevated in testes from $ER\alpha^{-/-}$ neonatal males (254). I performed real-time PCR analysis to test the hypothesis that these genes are regulated by estrogen through ERE-independent ERα mechanisms.

Statistical analyses revealed a significant effect of genotype on mRNA expression of StAR ($p<0.0001$) and all T synthesizing enzymes measured (P450scc, $p<0.0001$; $P450_{17\alpha}$, $p=0.0004$; 17β-HSD type III, $p=0.0038$; 3β-HSD type I, $p=0.0259$; and 3β-HSD type VI, $p=0.0005$) (Figure 18). Post hoc tests indicated that mRNA expression levels of StAR, 17β-HSD type III, 3β-HSD type I, and 3β-HSD type VI were significantly higher in testes from $ER\alpha^{-/-}$ mice compared to testes from both $ER\alpha^{+/+}$ and $ER\alpha^{-/AA}$ mice ($p<0.05$). P450scc mRNA expression was also significantly higher in testes from $ER\alpha^{-/-}$ mice compared to testes from both $ER\alpha^{+/+}$ and $ER\alpha^{-/AA}$ mice ($p<0.001$) and was lower in $ER\alpha^{-/AA}$ testes compared to $ER\alpha^{+/+}$ testes ($p<0.05$). $P450_{17\alpha}$ mRNA expression in $ER\alpha^{-/-}$ testes was approximately 1.4-fold higher than in $ER\alpha^{+/+}$ testes ($p>0.05$) and 1.8-fold higher than in $ER\alpha^{-/AA}$ testes ($p<0.001$). I additionally measured mRNA expression of two T metabolizing enzymes, P450 aromatase and 5α reductase and found no effect of genotype (Figure 19). These data demonstrate that ERE-independent ERα signaling mechanisms mediate estrogen inhibition of steroidogenic enzyme gene expression.

Enzyme activity

It has previously been demonstrated that the enhanced androgen biosynthesis in $ER\alpha^{-/-}$ Leydig cells is associated with increased enzyme activity of $P450_{17\alpha}$ and 17β-HSD (25). I was

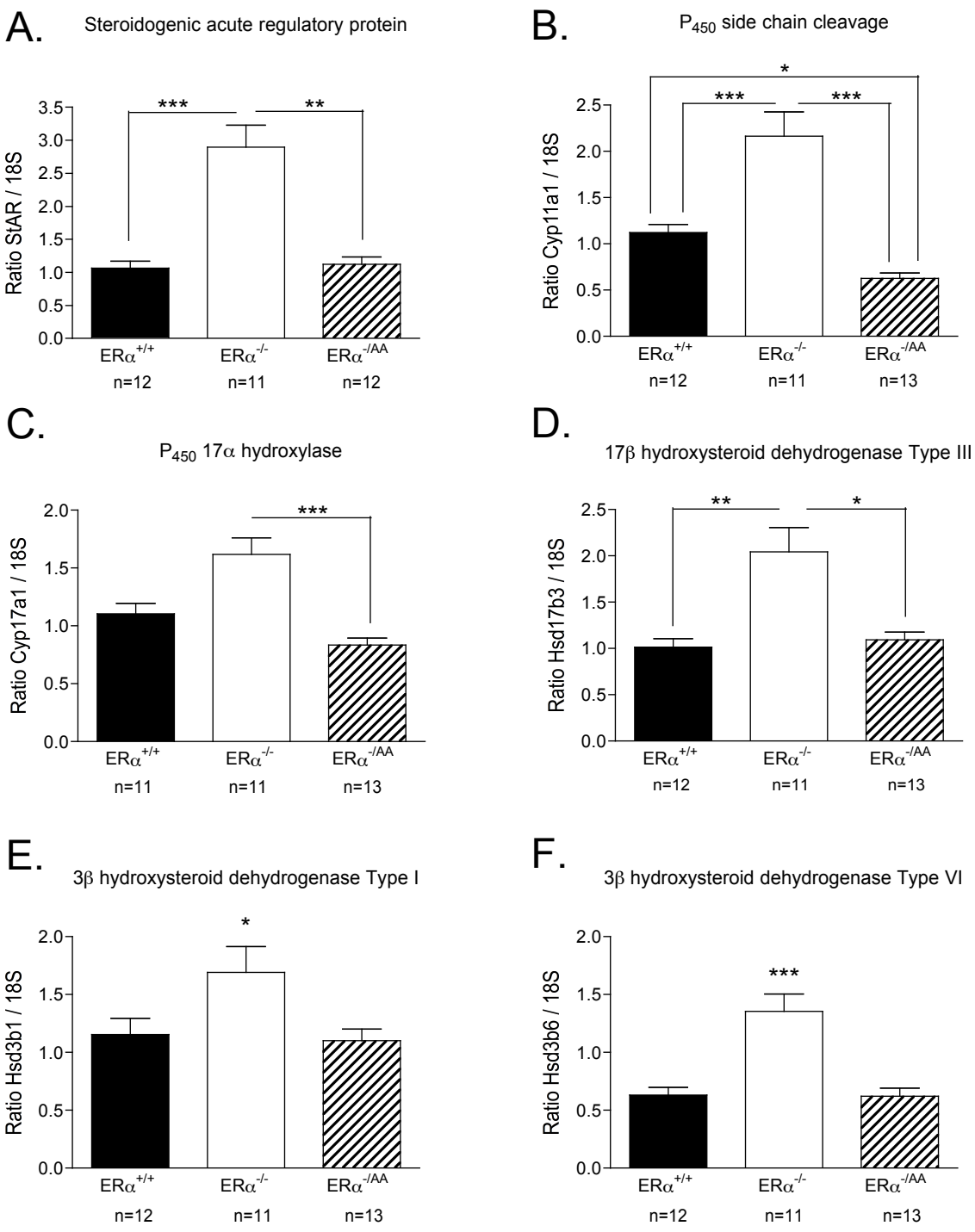


Figure 18. Steady state mRNA levels of androgen synthesizing enzymes. Real-time PCR analysis revealed that expression of StAR (A), P450scc (B), $P450_{17\alpha}$ (C), 17β-HSD type III (D), 3β-HSD type I (E) and 3β-HSD type VI (F) were elevated in the testes of $ER\alpha^{-/-}$ mice compared to $ER\alpha^{+/+}$ controls, but not in the testes of $ER\alpha^{-/AA}$ mice. *p<0.05, **p<0.01, ***p<0.001.

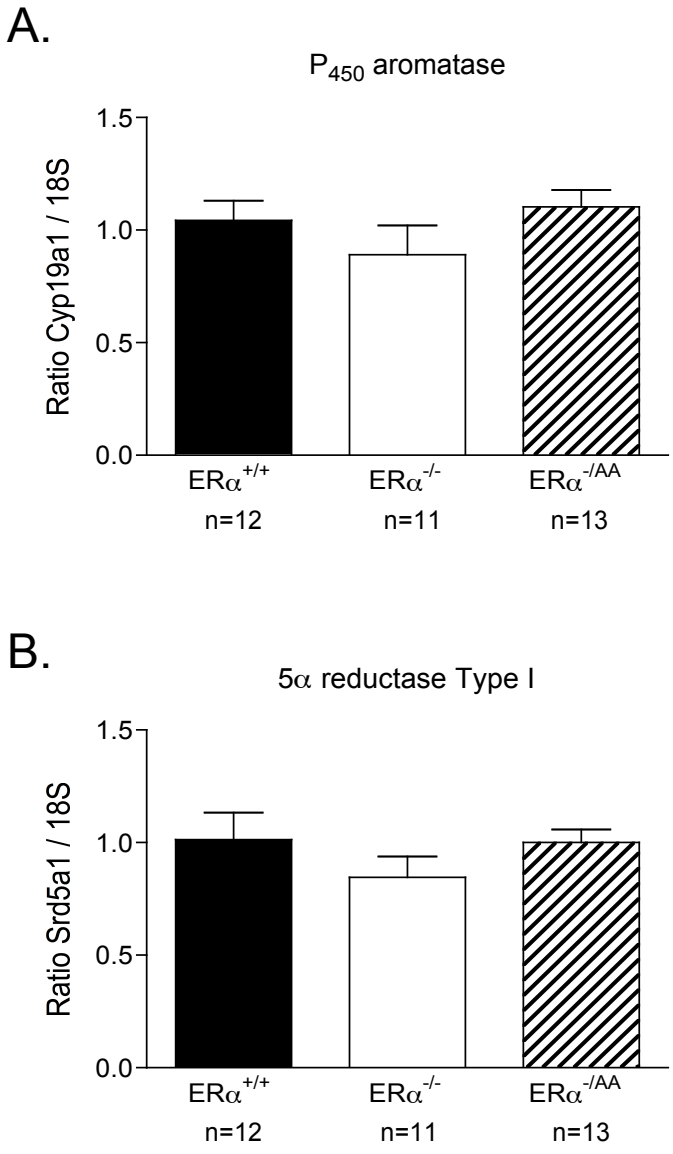


Figure 19. Steady state mRNA levels of androgen metabolizing enzymes. Real-time PCR analysis revealed that there was no effect of genotype on the expression of P450 aromatase (A) or 5α reductase (B).

therefore interested in knowing if steroidogenic enzyme activity is regulated by ERE-dependent or ERE-independent ERα mechanisms. A one-way ANOVA revealed a significant effect of genotype on the activity of $P450_{17\alpha}$ ($p=0.0001$), 3β-HSD ($p=0.0012$), and P450scc ($p<0.0001$), but there was no effect of genotype on 17β-HSD activity ($p=0.21$) (Figure 20). Consistent with the mRNA expression results described above, enzyme activities of 3β-HSD and P450scc were significantly elevated in $ER\alpha^{-/-}$ testes compared to $ER\alpha^{+/+}$ controls, and restored by the non-classical knock-in mutation in $ER\alpha^{-/AA}$ testes. Activity of $P450_{17\alpha}$ was significantly elevated in $ER\alpha^{-/-}$ testes ($p<0.001$), which is consistent with previous findings (25), but also elevated in $ER\alpha^{-/AA}$ testes compared to $ER\alpha^{+/+}$ controls ($p<0.001$). Together these results demonstrate that non-classical, ERE-independent ERα mechanisms are sufficient to mediate estrogen suppression of 3β-HSD and P450scc activity but not sufficient to mediate estrogen suppression of $P450_{17\alpha}$ activity.

Testis weights

Testes from both $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ mice weighed significantly less than testes from $ER\alpha^{+/+}$ controls at 4 to 5.5 months of age (Figure 21; one-way ANOVA with Newman-Keuls post hoc test, $p<0.05$).

Discussion

The goal of the present study was to determine if classical, ERE-dependent and/or non-classical, ERE-independent ERα mechanisms mediate estrogen regulation of gonadotropin secretion and T production in the male. I demonstrate here that the non-classical estrogen

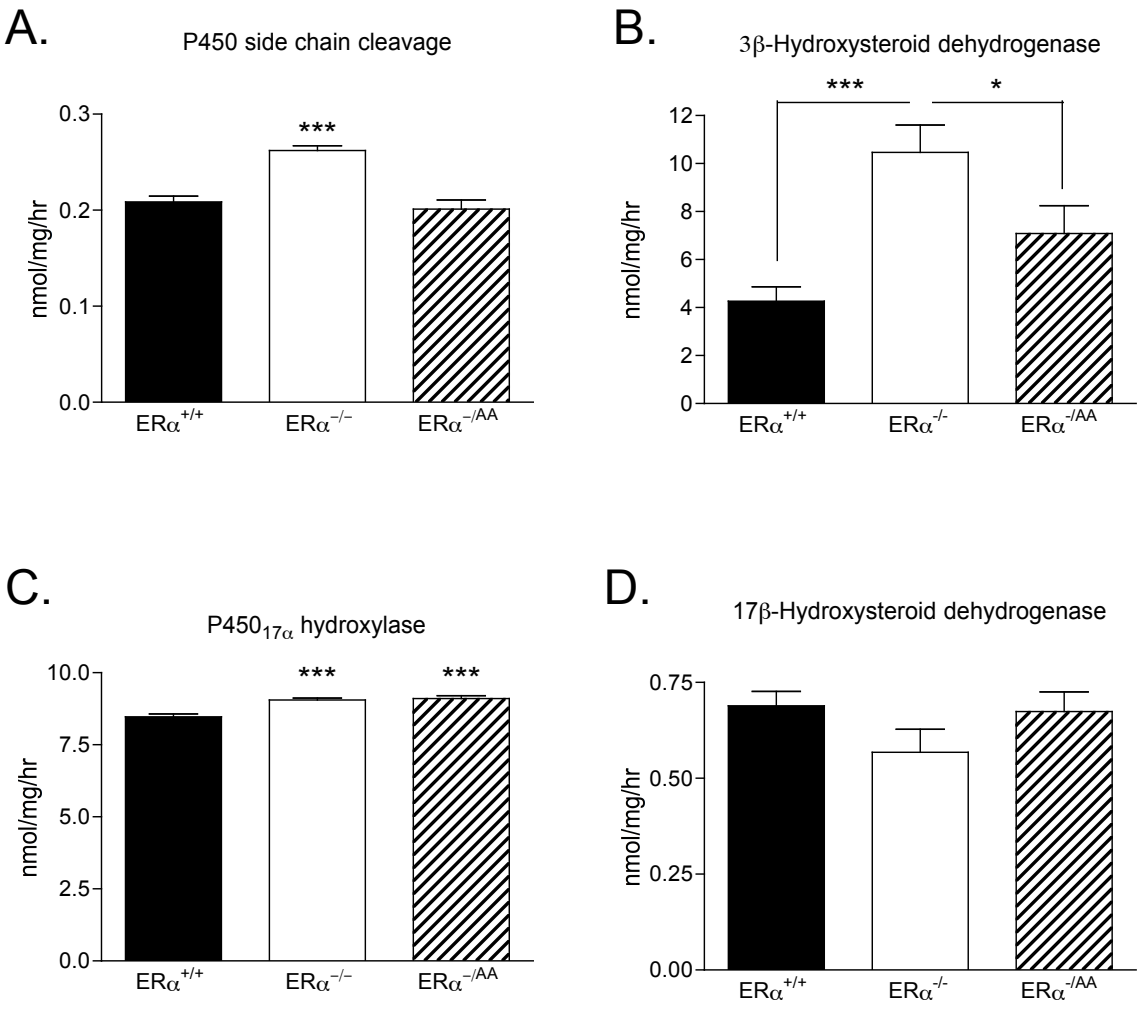


Figure 20. Activity of enzymes involved in androgen biosynthesis. The enzyme activities of P450scc (A), 3β-HSD type I (B), P450$_{17\alpha}$ (C), and 17β-HSD type III (D) were determined by conversion of radiolabeled substrates to their steroid hormone products. Data represent mean ± SEM. n=8 per group. *$p<0.05$, ***$p<0.001$.

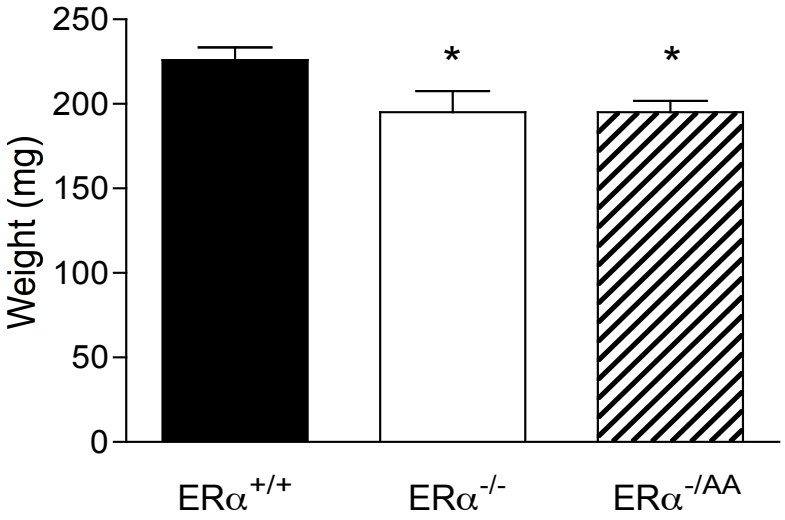


Figure 21. Testes weights. Total weight of left and right testes from ERα$^{-/-}$ and ERα$^{-/AA}$ mice is significantly less than that of testes from ERα$^{+/+}$ controls. n=10-13, *p<0.05.

receptor knock-in mutation in ERα$^{-/AA}$ male mice rescues the elevated serum T levels that result from ERα deletion in an LH-independent manner. Testicular T secretion *in vitro*, steroidogenic enzyme gene expression, and steroidogenic enzyme activity are all elevated in ERα$^{-/-}$ males and completely rescued in ERα$^{-/AA}$ males. These findings demonstrate that ERE-independent ERα signaling mechanisms are sufficient to mediate estrogen inhibition of androgen biosynthesis.

The non-classical estrogen receptor knock-in mouse model used in the present study provides a unique opportunity to examine the role of non-ERE-mediated mechanisms *in vivo*. While the mutant ERα cannot bind directly to DNA, it has been shown to interact with other transcription factors, such as Jun (80), and transcription through AP1 sites is preserved. Presumably, the mutant ERα in this mouse model can translocate to the cell membrane and mediate the rapid, non-genomic actions of estrogen as well. Therefore, it is important to note that my findings here do not distinguish between these two types of non-classical, ERE-independent mechanisms. Evidence from the teleost fish Atlantic croaker suggests that estrogens may inhibit androgens via a rapid, non-genomic mechanisms. The rapid, inhibitory effects of estrogens and xenoestrogens on androgen production in testicular cultures were blocked by treatment with transcription and translation inhibitors, and E_2-BSA was also capable of exerting inhibitory effects on androgen production, supporting a role for a membrane-associated estrogen receptor (260). Further analyses using the ERα$^{-/AA}$ mouse model will be useful in teasing apart the non-classical mechanisms of estrogen action in order to determine if estrogen inhibits androgen biosynthesis through ERE-independent genomic and/or non-genomic pathways.

ERα deletion increased expression of mRNAs encoding StAR and all the steroidogenic enzymes involved in T synthesis. My findings of elevated StAR, 17β-HSD type III, and $P450_{17\alpha}$ mRNA expression in adult ERα$^{-/-}$ males are consistent with the findings of Akingbemi and

colleagues (25); however, I additionally observed changes in P450scc, 3β-HSD type I, and 3β-HSD type VI. The mRNA expression levels of these genes were completely restored to wild-type levels in $ER\alpha^{-/AA}$ testes, indicating that ERE-independent ERα signaling mechanisms are sufficient to mediate estrogen suppression of steroidogenic enzyme gene expression. Interestingly, the promoter regions of StAR, $P450_{17\alpha}$, and P450scc have been reported to contain EREs (254), suggesting that estrogens may act directly on these gene promoters via a classical mode of action. Nonetheless, my findings demonstrate that estrogen can act independently of EREs. Indeed, the promoter regions of these genes contain a number of non-ERE binding sites that have been implicated in non-classical ERα signaling, such as Jun (318). For example, transcription factors such as C/EBP, Sp1, CREB/CREM, Fos and Jun can bind to the StAR promoter to regulate its transcription (319). More detailed molecular analyses will be required to determine the exact mechanisms by which estrogen can regulate the transcription of each of these genes independently of EREs.

Consistent with the increase in mRNA expression, ERα deletion resulted in increased activity of P450scc, 3β-HSD, and $P450_{17\alpha}$. Surprisingly, I did not observe a significant increase in 17β-HSD activity, which was previously demonstrated to be elevated in $ER\alpha^{-/-}$ testes; however, this may reflect differences in culture methods, as whole testicular tissue was cultured in the present study instead of isolated Leydig cells (25). Despite the lack of effect on 17β-HSD activity, the increased expression and activity of the other enzymes in the steroidogenic pathway are likely sufficient to confer enhanced T production. The non-classical knock-in mutation effectively rescued the enzyme activities of P450scc and 3β-HSD, suggesting that estrogen regulation of their activities is mediated by ERE-independent mechanisms. In contrast, the mutation did not rescue the activity of $P450_{17\alpha}$, suggesting that ERE-dependent mechanisms

may contribute to the suppression of its activity. Nevertheless, overall inhibition of androgen biosynthesis appears to be associated with suppression of steroidogenic enzyme activity via non-classical mechanisms.

In ERα$^{-/-}$ males, testes appear normal until puberty but begin to degenerate as early as 20-40 days of age and are atrophic by 150 days (16). As ERα$^{-/-}$ males age, back-pressure from luminal fluid retention and atrophy of the seminiferous epithelium cause testes weights to decline (120). In the present study, testes from both ERα$^{-/-}$ and ERα$^{-/AA}$ mice weighed significantly less than testes from ERα$^{+/+}$ controls, suggesting that ERE-dependent mechanisms are required for maintenance of normal testis structure and physiology. However, previous findings from my laboratory have demonstrated a transient rescue of seminiferous tubule morphology by the knock-in mutation; testes from younger ERα$^{-/AA}$ males (approximately 2-3 months) appear normal or with only mild dysmorphogenesis, and moderate to severe testis pathology does not arise until later ages (Jeffrey Weiss, unpublished observations). Histological data from ERα$^{-/-}$ and ERα$^{-/AA}$ testes confirmed that tubule morphology was disrupted at the ages studied (4 to 5.5 months) and are thus consistent with these earlier findings (data not shown).

Increased serum testosterone can be attributed to a direct effect of increased steroidogenesis or to an indirect effect of increased stimulation by pituitary LH. It does not appear that deletion of ERα disrupts negative feedback, causing a consequent rise in LH, as the present study and others fail to show elevated LH in ERα$^{-/-}$ males (16, 229). In contrast, others have reported approximately 2-fold greater LH levels in intact ERα$^{-/-}$ males; however, it is important to note that those levels are not as high as those in castrated wild-type males. Moreover, castration of ERα$^{-/-}$ males resulted in a significant rise in LH, suggesting that negative feedback on LH by T may be mediated at least in part by mechanisms independent of ERα (230).

Clearly ERα is critical for negative feedback in the female, as demonstrated by extremely high LH levels in ERα$^{-/-}$ female mice (231); however, the androgen receptor may play the predominant physiological role in the male (229). My results would suggest that AR, or perhaps ERβ, is sufficient to mediate negative feedback on LH in the absence of ERα.

In the present study, serum T levels were elevated in ERα$^{-/-}$ males despite normal serum LH levels, and testes from ERα$^{-/-}$ males produced elevated T levels *in vitro* without LH stimulation, or with equivalent LH stimulation, supporting the idea that ERα deletion increases serum T directly by enhancing the capacity for androgen biosynthesis by individual Leydig cells (25). Additional evidence from fetal ERα$^{-/-}$ mice, in which LH levels have not begun to exert stimulation on the testis, and neonatal ERα$^{-/-}$ males, in which no change in circulating LH was observed, suggest a direct inhibitory effect of endogenous estrogens (254). In rams, low level estradiol immunoneutralization also induces elevations in serum T, increased StAR mRNA levels, and increased 17β-HSD activity without affecting pituitary LH secretion (256). Finally, studies using adult Leydig cell cultures from gonadotropin-deficient mice reveal that regulation of 3β-HSD expression is independent of LH stimulation (257). Based on these data, I conclude that the enhanced steroidogenesis in ERα$^{-/-}$ mice and the rescue of normal steroidogenesis in ERα$^{-/AA}$ mice reflects a direct effect at the level of the testis.

As the present study examined testes from intact males, my findings demonstrate that physiological levels of endogenous estrogens inhibit T production via ERE-independent pathways, and presumably exogenous estrogens would exert their effects in a similar manner. Estrogens and xenoestrogens have received specific attention due to their detrimental effects on the reproductive tract and fertility in wildlife, laboratory animals, and humans (237, 320-324). Treatments with high levels of estrogenic compounds have been shown to decrease serum T and

intratesticular T content *in vivo* (235, 239-246), reduce Leydig cell T secretion *in vitro* (247-250), inhibit Leydig cell regeneration (251), and suppress expression of steroidogenic enzymes (252, 253). The ERα$^{-/AA}$ model may therefore provide a useful screening tool to determine whether specific estrogenic agents exert their effects through ERE-independent ERα signaling pathways.

As with all genetically engineered mouse models, one caveat of the ERα$^{-/AA}$ mouse model is that ERE-dependent signaling is absent throughout development and therefore one cannot discern whether ERE-independent ERα signaling contributes to estrogen's organizational effects, activational effects, or both. Estrogen is known to inhibit fetal Leydig cell development and function (237, 325), and treatment with pharmacological doses of estrogens can have permanent detrimental effects on the male reproductive system (240, 244, 245, 252, 320), supporting an organizational effect of estrogens. However, estrogens have also been shown to inhibit T production in adult wild-type animals, demonstrating that they exert activational effects as well (25). The creation of inducible knockout and knock-in mice would be a powerful tool for discriminating among temporally dissociable effects of estrogen action on steroidogenesis.

ERE-independent ERα signaling also appears to play a role in the regulation of serum FSH levels, as demonstrated by significantly lower levels in intact ERα$^{-/AA}$ males compared to both ERα$^{+/+}$ and ERα$^{-/-}$ males. This is particularly interesting because both copies of ERα are capable of signaling via non-ERE mechanisms in wild-type animals, yet the AA mutation has a significant suppressive effect in the absence of ERE-dependent pathways. This suggests that perhaps ERE-dependent mechanisms antagonize the ERE-independent mechanisms that mediate E_2 effects on FSH. In support of my findings, transcriptional repression of the ovine FSHβ gene by E_2 appears to be mediated via receptor-protein interactions with basal transcription factors,

independent of direct DNA binding by ER (326). Specifically, oFSHβ transcription can be stimulated by c-Jun and c-Fos proteins via two AP-1-like sites in the oFSHβ proximal promoter, which appear to be important for the regulation of FSH production *in vivo* (327). It remains a possibility that ERE-independent ERα signaling does not act on FSH synthesis directly but rather through another mechanism, such as by increasing testicular inhibin or reducing activin. Further studies will be required to determine the mechanisms involved in the suppression of serum FSH in $ER\alpha^{-/AA}$ males.

In conclusion, this study demonstrates for the first time an ERE-independent ERα-mediated inhibitory effect of endogenous estrogens on testicular steroidogenesis in the adult mouse *in vivo*. These findings will ultimately be helpful in understanding how estrogens contribute to T's influence on the development and maintenance of the male reproductive tract, and may be useful for generating new strategies to treat testicular development disorders and adult male infertility, particularly those that arise from exposure to environmental estrogenic agents.

V. SUMMARY AND DISCUSSION

Overview

Successful reproduction in the male depends upon normal T secretion, in large part, to masculinize the male brain during development, and to stimulate spermatogenesis and sexual behaviors in adulthood, which ultimately allows for passing on of gametes and generating progeny. Therefore, disruption of normal T secretion, or of the downstream mechanisms underlying T's actions, inevitably impairs overall reproductive function. The experiments described in this thesis have focused on two important aspects of male reproductive function: sexual behavior and regulation of the reproductive hormone axis. Steroid regulation of sexual behavior has been studied for decades; however, the molecular mechanisms of its action are only recently being identified. For example, early research identified that T enhances neuronal activity and consequently sexual activity, but little research has been devoted to the cellular and molecular mechanisms by which T exerts these effects. The experiments in Chapter II were therefore designed to test the hypothesis that K^+_{ATP} channels serve as a mechanism by which T controls neuronal activity and sexual behavior in male rats. Previous research has also identified a prominent role for estrogen and ERα signaling in male sexual behavior, however, ERα participates in a variety of signaling pathways that have not been examined in the context of sexual behavior. Chapter III therefore examined the molecular mechanisms of ERα signaling underlying estrogen stimulation of male sexual behavior in the mouse. Based on the results presented in Chapters II and III, I conclude that steroid actions on male sexual behavior are mediated in part through inhibition of K^+_{ATP} channels and through activation of ERE-dependent ERα signaling mechanisms.

Given testosterone's importance in regulating so many key features of male reproductive function, understanding the regulation of its production is critical for understanding how intrinsic

and extrinsic factors ultimately affect male physiology and behavior. Both endogenous and exogenous estrogens have been demonstrated to exert inhibitory effects on T secretion from the testes. Interestingly, both estrogen deficiency and inappropriate estrogen exposure can have detrimental effects on the male reproductive tract, spermatogenesis, steroidogenesis, and behaviors. Much of our understanding of the mechanisms of estrogen action has been derived from the use of gene knockout models, and it is now known that ERα largely, if not solely, mediates estrogen's inhibitory effects on T biosynthesis. However, the relative roles of classical and non-classical ERα signaling pathways have not been identified. The experiments in Chapter IV were therefore conducted to provide a better understanding of the mechanisms by which endogenous estrogens suppress androgen biosynthesis in the male mouse. My results have led me to conclude that T production is inhibited through ERE-independent ERα signaling mechanisms.

This dissertation has effectively described two novel molecular mechanisms of steroid hormone action *in vivo* and contributes to our knowledge of how these steroids regulate male reproductive function.

Cellular & molecular mechanisms underlying steroid regulation of sexual behavior

Chapter II of this dissertation established for the first time that neural K^+_{ATP} channels are inhibited by T and demonstrated that they play a role in sexual behavior. Experiments described in this chapter demonstrated that T inhibits mRNA expression of the K^+_{ATP} channel subunit Kir6.2 and that central blockade of K^+_{ATP} channels stimulates copulation independently of testicular testosterone. These findings show that the K^+_{ATP} channel serves as a potential mechanism by which testosterone increases neuronal activity and elicits male sexual behavior.

In Chapter III of this dissertation I identified a second mechanims of steroid regulation sexual behavior, namely that E_2 stimulation of copulation requires classical, ERE-dependent signaling through ERα. Experiments in this chapter demonstrate that non-classical signaling was insufficient to restore sexual behavior in the absence of ERα-DNA binding. Although there is some evidence for rapid actions of E_2 on male sexual behavior, the present findings are consistent with previous *in vivo* studies that demonstrate the importance of genomic actions of E_2 on masculine sexual behavior, and extend this idea to include ERE-dependent genomic actions of E_2. Thus, the gene targets of E_2 action that contribute to the expression of behavior likely contain EREs.

Although the findings from Chapters II and III are taken from different species, I propose that these molecular mechanims of steroid hormone action may be linked (Figure 22). Specifically, I propose that T inhibits K^+_{ATP} channel subunit expression and consequently male sexual activity via classical, ERE-dependent ERα mechanisms. While future studies will need to examine the effects of T metabolites DHT and E_2 (or other specific receptor agonists) on Kir6.2 mRNA expression, a classical, ER-mediated effect is supported by findings that, (1) the majority of T's central effects on male sexual behavior are via aromatization to E_2 (3), (2) copulation requires slow, genomic effects of steroids (126, 133), and (3) one ERE is present in the Kir6.2 promoter (328). However, recent studies from my laboratory have demonstrated that estrogen actually stimulates Kir6.2 mRNA expression in the hypothalamus of female rats (11), which is in direct opposition to my findings for T in the male, but it is possible that sex differences in estrogen regulation of Kir6.2 mRNA expression exist.

Interestingly, the defects in sexual behavior in castrated male rats (274) and in ERαKO male mice (18) have been attributed to defects in central dopamine release, and K^+_{ATP} channels

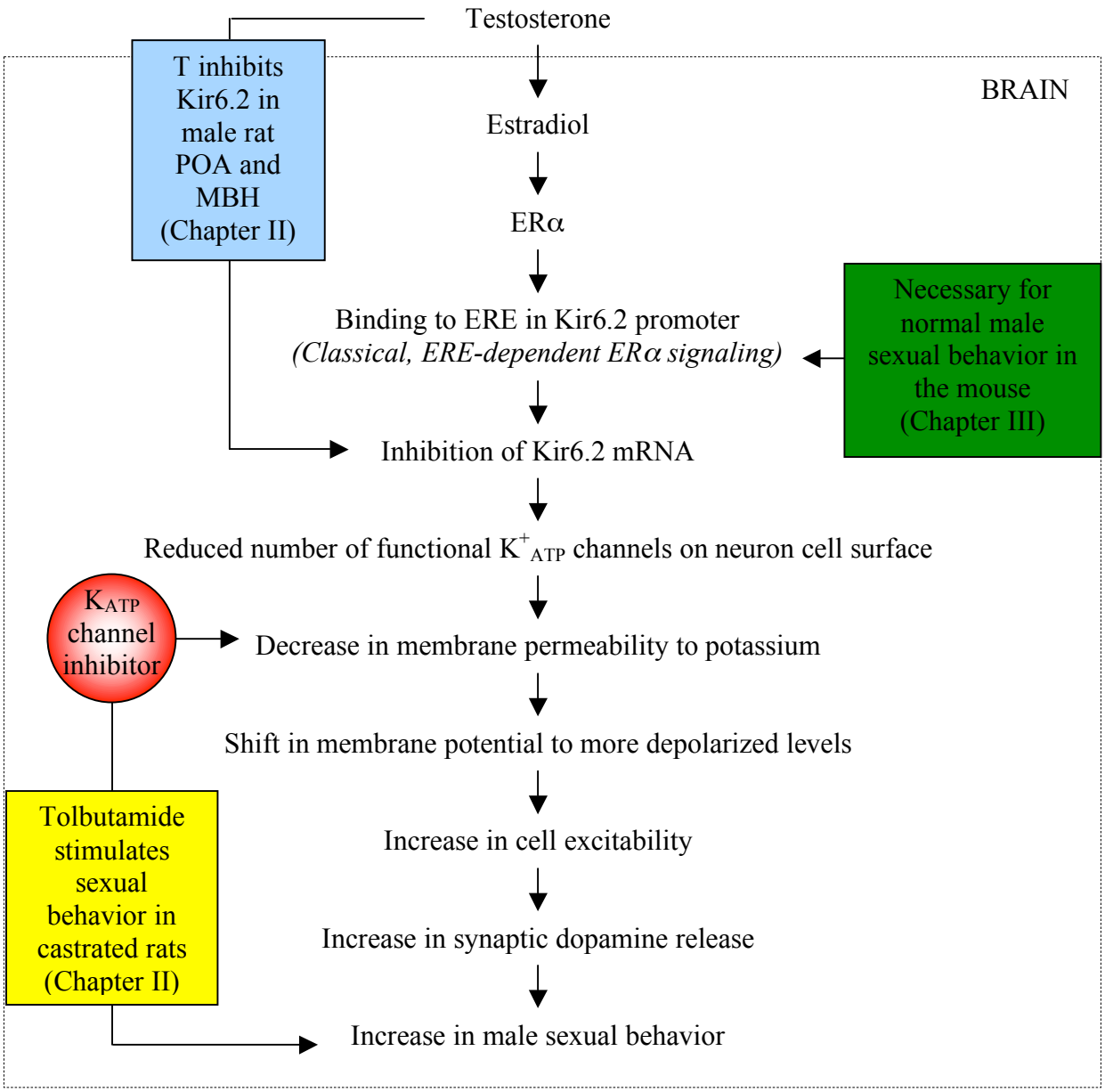


Figure 21. Proposed model of steroid regulation of male sexual behavior.

have been implicated in mediating dopamine release (10, 13, 14, 281). Therefore, if K^+_{ATP} channels are indeed regulated by ERα-dependent mechanisms, in the absence of ERα or ERα-stimulation by E_2, Kir6.2 mRNA expression would be expected to be elevated, causing reduced cell excitability and neurotransmitter release from synaptic terminals. Future studies may therefore wish to address the following questions: Does T inhibit Kir6.2 mRNA expression in the mouse POA/MBH as it does in rats? Is K^+_{ATP} channel subunit expression altered by ERα deletion? Can tolbutamide restore sexual behavior to castrated male mice as it does in castrated male rats? Can tolbutamide restore sexual behavior to ERαKO males? Extending the present findings from the rat to the mouse would provide more opportunities to examine the exact mechanisms underlying T inhibition of K^+_{ATP} channels through the use of knockout and knock-in mouse models.

While testosterone has been shown to modulate K^+_{ATP} channels in non-neuronal tissues, this study is the first to demonstrate that T regulates channel subunit expression. In cardiac tissue, supraphysiological concentrations of T induce relaxation of human radial arteries by opening K^+_{ATP} channels (329), and activation of cardiac K^+_{ATP} channels appears to be associated with T-induced cytoprotection (330). Interestingly, K^+_{ATP} channels are also localized to penile tissue and have been implicated in mediating the vasorelaxant effects of T in human corporal smooth muscle (331), suggesting that this may be an additional peripheral mechanism by which T controls copulation. Within the gonad, recent evidence suggests that T closes K^+_{ATP} channels and depolarizes Sertoli cell membranes through the PLC-PIP2 pathway (332). Therefore, T's effects on neuronal K^+_{ATP} channels in the POA may not be limited to inhibition of Kir6.2 subunit mRNA expression. Thus, whether T modulates neural K^+_{ATP} channels by other mechanisms, and whether T inhibits K^+_{ATP} channel subunit expression in other tissues, remains to be determined.

The experiments in Chapter II identified K^+_{ATP} channels as mediators of T's activational effects on male sexual behavior, but do not address whether they also play a role in T's permanent, organizational effects on sexual behavior. T exposure during critical periods of pre- and perinatal development is both necessary and sufficient to masculinize sexual behavior. Previous findings from my laboratory have in fact demonstrated that prenatal androgen exposure suppresses Kir6.2 expression in the female rat POA (Eileen Foecking, unpublished observations), and similar regimens have been shown to induce masculine sexual behavior in females (333). Thus, it is possible that T may permanently masculinize the circuitries responsible for sexual behavior in part by inhibiting K^+_{ATP} channel subunit expression. It will be interesting to test whether neonatal castration has lasting effects on Kir6.2 expression in adulthood, and whether there is a sex difference in K^+_{ATP} channel subunit expression.

Similarly, the experiments in Chapter III unfortunately do not distinguish between a requirement for ERE-dependent ERα signaling during development or in adulthood, since one caveat of all genetically engineered mouse models is that the gene deletion or mutation is present throughout the animals' lifetime. It is well established that both organizational and activational effects of estrogen are critical for male sexual behavior (270, 334). During neonatal development, estrogen exerts masculinizing effects on the brain and behavior via activation of an estrogen receptor (270, 335-337). ER antagonists and ER antisense mRNA treatments have been shown to block the masculinizing effect of neonatal T exposure in the female (338, 339). However, it is not entirely clear whether the ER that mediates E_2's effects during development is ERα. E_2's activational effects through ERα are demonstrated by the observation that ER antagonists prevent the restoration of copulation by T (138). Of note, Rissman and colleagues have suggested that, because ERαKO male mice could be induced to copulate following

administration of the dopamine agonist apomorphine, ERα may not be required for the development of neural circuitries controling sexual behavior (18). The creation of inducible knockout and knock-in mice would be a powerful tool for teasing apart the temporal effects of estrogen action on male sexual behavior.

While the data presented here support a role for both K^+_{ATP} channels and ERE-dependent ERα signaling mechanisms in male sexual behavior, it is unknown whether these are common mechanisms by which steroids control behavior of both sexes. Estrogen and progesterone are clearly necessary for female sexual behavior, however, these steroids were shown to stimulate expression of Kir6.2 mRNA expression (11), which, based on my model, would suppress cell excitability. It is possible that K^+_{ATP} channels do not play a role in female sexual behavior, or that E and P reduce excitability of neurons that are inhibitory to female sexual behavior. It would therefore be interesting to examine the effects of pharmacological K^+_{ATP} channel inhibitors on female sexual behavior both in intact and ovariectomized females.

My findings that non-classical ERα signaling mechanisms were insufficient to mediate E_2 stimulation of copulation in the male raise the possibility that classical, ERE-dependent mechanisms are also required for estrogen regulation of sexual behavior in the female. Preliminary studies from my laboratory have indicated that while $ER\alpha^{-/AA}$ females are unreceptive to males and do not show lordosis behavior, they do display significantly less rejective behavior (e.g. kicking, fleeing, and rearing) and more proceptive behavior (e.g. approach of males, pausing for an attentive male) than $ER\alpha^{-/-}$ counterparts (Mariana Jimenez, unpublished observations). These data indicate that non-classical ERα signaling is sufficient to maintain proceptive components of female sexual behavior in the absence of classical ERα-DNA binding, and that paracopulatory and copulatory behaviors may be mediated by different

molecular mechanisms. Furthermore, these findings suggest that E_2 regulation of sexual behavior in the male and female are qualitatively different.

The data presented in this dissertation from rodent studies may provide insight into potential strategies to treat sexual dysfunction in humans. The proposed roles for K^+_{ATP} channels and ERα signaling mechanisms in dopamine signaling in the rodent brain (described above) is particularly relevent to human health, based on evidence that dopamine also enhances sexual behavior in men. For example, L-DOPA, the precursor to dopamine, was shown to increase libido and sexual potency in Parkinson's disease patients, independent of improvements in motor function (340-342). The dopamine agonist apomorphine has also been used clinically to treat human erectile function (343). Furthermore, dopamine antagonists used in the treatment of schizophrenia have been reported to cause side effects including sexual dysfunction and decreased libido (344, 345). Therefore, dysregulation of neural K^+_{ATP} channel function or ERα signaling could potentially have a negative impact on human sexual behavior.

Hypothalamic K^+_{ATP} channels couple metabolic signals to cell excitability (7), and evidence suggests that sexual dysfunction is often associated with metabolic diseases, such as diabetes (287, 288). For example, sexual behavior is severely disrupted as a result of spontaneous or experimentally-induced diabetes in male rats (289-294) and some defects may be due, at least in part, to a central neuropathy (291). Thus, K^+_{ATP} channels may represent a common pathway by which T and metabolic signals modulate male sexual behavior. Interestingly, the Kir6.2 gene is an established type II diabetes-susceptibility variant and is implicated in the pathogenesis of multifactorial type II diabetes (346). Although it is unknown whether Kir6.2 variants cause abnormal functioning in neuronal K^+_{ATP} channels, dysregulation of

androgen target neurons in circuitries governing sexual motivation could potentially provide an explanation for the loss of libido in patients with type II diabetes.

In Chapter II, central infusions of the K^+_{ATP} channel inhibitor tolbutamide were shown to stimulate sexual behavior in castrated male rats. Tolbutamide has been used clinically to treat symptoms of type II diabetes, specifically to increase insulin secretion from pancreatic β-cells, and thus the present findings raise the intriguing possibility that tolbutamide, or similar K^+_{ATP} channel inhibitors, may potentially have other pharmacological uses. However, it is important to note that tolbutamide only had an effect in the absence of T in the present studies, and presumably tolbutamide-treated patients have relatively normal circulating levels of this steroid. Furthermore, the tolbutamide was administered centrally in these studies. Interestingly, Benelli and colleagues have demonstrated stimulation of sexual behavior in intact rats by systemic administration of the K^+_{ATP} channel inhibitor glibenclimide (178). Whether K^+_{ATP} channel inhibition could have similar effects in humans remains to be determined.

ERE-independent ERα signaling mechanisms in male reproductive function

The $ER\alpha^{-/AA}$ model provides a unique opportunity for characterizing classical and non-classical ERα signaling mechanisms in the brain and behavior. As estrogen regulation of physiology and behaviors requires fine-tuned control, it is perhaps not surprising that ERE-independent mechanisms are sufficient to mediate estrogen's actions in some systems but not others (Figure 23). As discussed above, Chapter III of this dissertation demonstrated that non-classical signaling was insufficient to restore copulation in the absence of ERα-DNA binding. In contrast to the requirement for classical ERα mechanisms in sexual behavior, experiments in Chapter IV demonstrate that non-classical, ERE-independent ERα mechanisms were sufficient

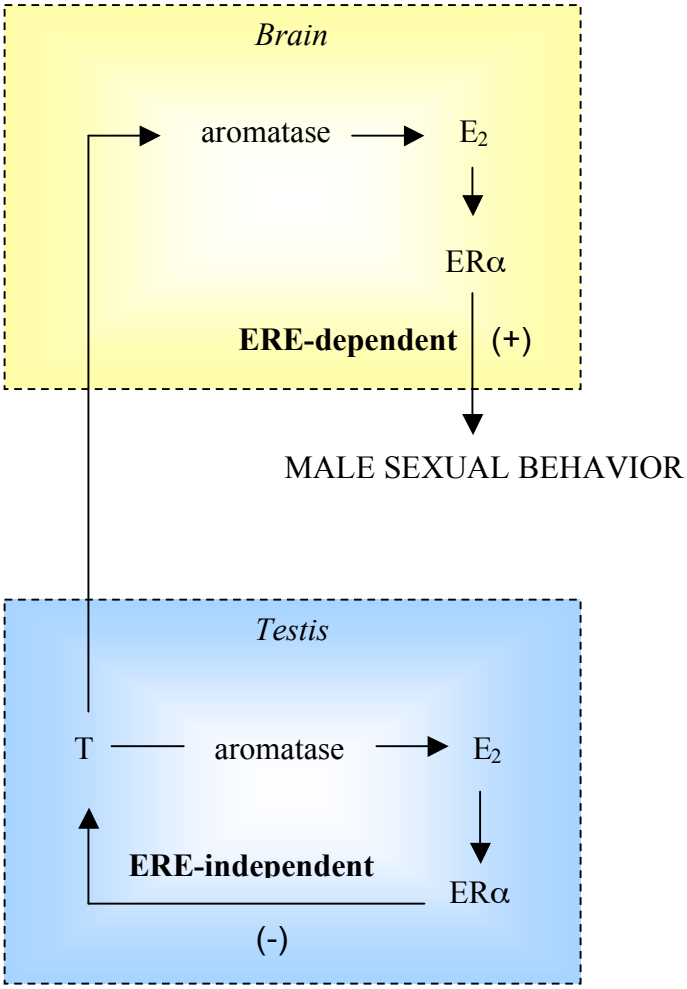


Figure 23. ERα signaling mechanisms in male reproductive function. Testosterone (T) produced from Leygdig cells in the testis is converted to estradiol (E_2) by the enzyme aromatase. E_2 inhibits T biosynthesis via ERE-independent ERα signaling mechanims. T also enters the bloodstream and is aromatized to E_2 within the brain. E_2 stimulates male sexual behavior via ERE-dependent ERα signaling mechanisms.

to restore serum testosterone levels, and may additionally play a role in the regulation of FSH. Experiments in Chapter IV were therefore conducted to test the hypothesis that non-classical, ERE-independent ERα mechanisms mediate estrogen inhibition of androgen biosynthesis. Specifically, the non-classical ERα knock-in mutation in $ER\alpha^{-/AA}$ mice rescued testicular T secretion, steroidogenic enzyme gene expression, and steroidogenic enzyme activity.

As mentioned previously, one caveat of the $ER\alpha^{-/AA}$ mouse model is that the gene mutation is present both during development and in adulthood, and therefore one cannot determine whether ERE-independent ERα signaling mechanisms are mediating organizational and/or activational effects of E_2 on androgen biosynthesis. It is well known that endogenous E_2 inhibits T production both during fetal and neonatal development and in adulthood (25, 254). An organizational effect of E_2 is supported by studies in which exposure to high levels of estrogenic agents pre- or perinatally cause permanent changes in testicular function that persist into adulthood. However, estrogens have also been shown to inhibit T production in adult wild-type animals, suggesting that they exert activational effects as well. The creation of inducible knockout and knock-in mice would be a powerful tool for discriminating among temporally dissociable effects of estrogen action on steroidogenesis.

Another caveat of the $ER\alpha^{-/AA}$ model is that it does not distinguish between the variety of non-ERE-mediated mechanisms that have been proposed for E_2 action. The mutant ERα in the $ER\alpha^{-/AA}$ model can alter gene transcription at non-ERE sites by tethering to other transcription factors such as Jun (80), but recent evidence suggests that the mutant ERα in the $ER\alpha^{-/AA}$ model is also capable of translocating to the plasma membrane and mediating nongenomic effects of E_2 (Jeffrey Weiss, personal communication). The mutant ERα's association with the plasma membrane is in fact expected, given that the AA mutation is specific to the DNA-binding domain

of ERα and does not disrupt the localization sequences that have been shown to mediate palmitoylation within the E domain of ERα and facilitate caveolin-1 association, subsequent membrane localization, and steroid signaling (347). Thus, it is not clear whether the non-classical, ERE-independent ERα signaling mechanisms that mediate E_2 inhibition of T production are genomic or non-genomic. The finding that E_2 suppresses steroidogenic enzyme gene expression in $ER\alpha^{-/AA}$ testes might suggest that the ERE-independent mechanisms involved are genomic. Detailed analyses of the promoter regions of these genes will be helpful in determining the exact signaling mechanisms that may mediate E_2's effects on their expression. The virtual absence of sexual activity in $ER\alpha^{-/AA}$ males indicates that none of the non-classical pathways of E_2 action is sufficient to maintain normal masculine sexual behavior in the absence of ERE-dependent pathways. Future studies will attempt to address the issue of genomic vs. non-genomic ERE-independent ERα signaling by utilizing compounds that are essentially confined within the cytoplasm and comparing their effects to E_2 in the $ER\alpha^{-/AA}$ mouse.

Unfortunately, the apparent infertility of heterozygous females ($ER\alpha^{+/AA}$) currently precludes the generation of $ER\alpha^{AA/AA}$ animals, and we are therefore unable to study the AA mutation in the homozygous state (26). While it could be argued that the presence of only one mutant ERα allele may be insufficient to elicit significant effects, several studies of $ER\alpha^{-/AA}$ mice support the idea that one mutant allele is enough to at least partially rescue the phenotypes generated by the deletion of the wild-type ERα. For example, the knock-in mutation is sufficient to restore androgen biosynthesis (Chapter IV), trabecular bone mineral density (124), negative feedback on gonadotropin secretion in the female (125), and testicular degeneration and epididymal dysfunction (Jeffrey Weiss et al., unpublished observations). However, different physiological processes can have different gene expression requirements, and two copies of the

mutant ERα may be necessary for the restoration of some $ER\alpha^{-/-}$ phenotypes. The issue of gene dosage also warrants attention, given that $ER\alpha^{+/AA}$ females display a strong reproductive phenotype despite the presence of one wild-type allele. For that same reason, future studies may also wish to more closely examine the phenotypes of $ER\alpha^{+/AA}$ males, although they appear to be fertile.

Finally, some of the intracellular actions of the mutant ERα in $ER\alpha^{-/AA}$ mice remain to be determined. For example, ERα and ERβ are known to form heterodimers, but it is not clear whether the AA mutant can interact with ERβ, and if it does, whether the heterodimer is transcriptionally active. Therefore, phenotypes in the $ER\alpha^{-/AA}$ mouse may in fact be due to E_2 stimulation of ERβ. Nonetheless, the studies presented here are a first step toward understanding the molecular actions of estrogen in the male and highlight the differential role for classical and non-classical mechanisms in physiology and behavior.

Summary

This dissertation expands our current understanding of the cellular and molecular mechanisms of steroid hormones in the regulation of male reproductive physiology and behavior. Previous studies have identified a role for T, E_2, and enhanced neuronal activity in sexual activity. This thesis establishes for the first time the role of K^+_{ATP} channels as mediators of T's stimulatory effects on neuronal activity and sexual behavior, and additionally identifies a requirement for ERE-dependent ERα signaling in mediating E_2's stimulatory effects on behavior. It also establishes that ERE-independent ERα signaling mechanisms mediate E_2's inhibitory effects on T production. Understanding the distinct molecular mechanisms of steroid action in specific target tissues and physiological systems will ultimately provide new

possibilities for the development of pharmacological therapies that treat testicular or sexual dysfunction in the male.

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APPENDIX I: THE ROLE OF CLASSICAL AND NON-CLASSICAL ERα SIGNALING IN STRESS RESPONSIVENESS, ANXIETY, AND DEPRESSION

Abstract

The expression of stress, anxiety, and depression is sexually dimorphic in humans and rodents. Females respond more robustly to stressors and develop anxiety and depression disorders more frequently than males. Varying levels of estrogen during fetal development and throughout adulthood are believed to cause these sex differences. However, relatively little is known about the effects of estrogen on these behaviors in the male. The goal of the present study was to examine the effects of ERα deletion on basal and stress-induced corticosterone secretion, anxiety-like behavior, and depressive-like behavior in male mice. I additionally utilized non-classical ERα knock-in mice ($ER\alpha^{-/AA}$), in which the mutant ERα can only participate in ERE-independent signaling pathways, to examine the relative roles of classical and non-classical ERα signaling mechanisms *in vivo*. I found that basal corticosterone levels were elevated in $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males, similar to that observed in female mice, suggesting that E_2 may masculinize the HPA axis via classical ERα signaling pathways. $ER\alpha^{-/AA}$ mice also display an enhanced response to restraint stress, suggesting that non-classical ERα pathways stimulate corticosterone secretion in the absence of ERα-DNA binding. Elevated plus maze, open field, and marble-burying tests revealed that ERα does not contribute to anxiety-like behavior, and the forced-swim test revealed that ERα does not contribute to depressive-like behavior in the male. Although stress is closely associated with anxiety and depression, my findings suggest that E_2 regulates these responses independently. Together these data contribute to our understanding of the molecular mechanisms underlying estrogen regulation of stress and affective behaviors in the male.

Introduction

Activity of the hypothalamic-pituitary-adrenal (HPA) axis is sexually dimorphic and is attributed, in part, to circulating gonadal steroid hormones (348), (349-355). Adult female rodents display enhanced basal and stress-induced corticosterone secretion compared to males (349, 353-358). In males, testosterone (T) inhibits HPA axis activity, whereas in females, estrogens enhance HPA axis activity (354, 355, 357, 359).

Other evidence suggests that the sexual dimorphism in corticosterone release is due, at least in part, to organizational effects of estrogen during critical periods of development (358, 360). Neonatal estrogen treatment in female rats effectively masculinized a number of neuroendocrine mechanisms related to HPA axis function (358), and male rats treated during prenatal and neonatal development with the aromatase inhibitor ATD displayed elevated basal and stress-induced corticosterone levels (360), suggesting that early estrogen exposure acts to masculinize HPA axis activity. Based on this evidence, I hypothesized that blocking estrogen signaling through ERα deletion would similarly feminize the male HPA axis. Interestingly, stress responsiveness has not been examined in any of the ER knockout mouse models to date.

Affective behavior in rodents can be altered by HPA axis activity (361); however, whether there is a relationship between estrogen's effects on the HPA axis and affective behavior is not entirely clear. Anxiety and depression are sexually dimorphic and regulated by estrogen, but few studies have addressed the effect of estrogen on these behaviors in the male, and those that do have produced conflicting results. In males, estradiol benzoate has been shown to increase anxiety in male rats (362), but have no effect in male mice (363), whereas estradiol (E_2) has been shown to reduce anxiety of male mice, but only in some tests (364). In the forced-swim test, castration induces depressive-like behavior, and E_2 has an acute antidepressant effect (365).

Recent evidence also demonstrates that adult male mice treated for five days with the ER antagonist tamoxifen displayed significantly more depressive-like behavior than controls in the forced-swim test, as indicated by more time spent immobile, even four weeks after the drug treatment (366). However, aromatase knockout mice (ArKO) have been reported to display normal levels of anxiety- and depressive-like behavior, indicating that estrogen deficiency has no affect on these behaviors (367). The role of ERα in mediating estrogen effects on mood and affect is also unclear. While ERβ appears to play a predominant role in the regulation of anxiety and depression (368), other evidence suggests that ERα makes some contributions to these behaviors. For example, the ERα-specific agonist PPT increased anxiety in adult rats of both sexes (369). ERαKO males have been reported to display increased (181), decreased (180), or normal anxiety (370). Female ERαKO mice demonstrate increased anxiety compared to wild-type counterparts (71). Of note, ERα mRNA expression is lower in the medial posterodorsal amygdala of Flinders Sensitive Line rats, a genetic model of depression (371), and is reduced in several brain regions of patients with major mental illness (372, 373), and ERα gene polymorphisms are associated with anxiety disorder, suicidal behavior, major depressive disorder, bipolar disorder, and psychosis (374-376). However, there is no evidence to date of the effect of ERα gene deletion on depressive-like behavior in male mice. I therefore chose to examine anxiety- and depressive-like behavior in male ERαKO mice.

As ERα has been shown to participate in a number of signaling pathways, the present studies also utilize the non-classical ERα knock-in mouse ($ER\alpha^{-/AA}$) to further investigate the relative roles of ERE-dependent and ERE-independent ERα signaling to stress responsiveness, anxiety, and depression in the male.

Materials and Methods

Animals

ERα null (ERα$^{-/-}$) and ERα$^{+/AA}$ mutant mice were generated as previously described in Chapter III. Of note, C57Bl/6 mice have been characterized as having an intermediate level of anxiety (i.e. not particularly anxious compared to other strains) (377). Adult male mice were housed under a 12:12 light-dark cycle with food and water available *ad libitum*. Males were castrated under isoflurane anesthesia and received one 10 mm Silastic capsule (o.d. 2.16 mm, i.d. 1.02 mm; Dow Corning Silastic, Helix Medical Inc., Carpinteria, CA) packed with 5 mm of crystalline testosterone (Sigma T-1500, St. Louis, MO) implanted subcutaneously into the midscapular region. This castration and hormone replacement was conducted to normalize serum T levels across genotypes, as intact ERα$^{-/-}$ males have been shown to have significantly elevated serum T (16, 19, 255). These T capsules produced average serum T levels of 1.08 ± 0.07 ng/ml, which are comparable to those observed in intact wild-type males (0.91 ± 0.36 ng/ml). Following surgery, mice were individually housed and tested a minimum of one week after isolation. Anxiety tests were performed in the same room where mice were housed to minimize changes in outside disturbances such as motion or noise. Stress and depression tests were conducted in a nearby procedure room to keep test animals isolated from the rest of the colony.

Restraint Stress

Restraint is a mixed physiological and psychological stress that has been shown to significantly induce secretion of the stress hormone corticosterone (378). To determine basal serum corticosterone levels, mice were quickly removed from their cages and blood was immediately obtained from the submandibular region using a sterile GoldenRod animal lancet

(Medipoint, Inc., Mineola, NY) during the light phase of the light:dark cycle, between 1000h and 1200h. The subject was then immediately placed into a ventilated plastic restraint tube that limited movement. After thirty minutes of restraint, mice were removed and a second blood sample was obtained from the opposite submandibular area. Each restraint tube was thoroughly cleaned between animals. Blood was centrifuged and serum collected and stored at -20°C until radioimmunoassay.

Elevated Plus Maze

The elevated plus maze (EPM) is a standard test for anxiety-like behavior that capitalizes on a rodent's unconditioned aversion to heights and open spaces (379). The EPM used in these tests was raised 41 cm off the floor and each of the arms was 33 cm in length and 7.5 cm wide. The closed arms each had 18 cm high black walls. The mouse is placed on the center of the platform facing an open arm and allowed to explore the maze for five minutes. Each test was recorded with a video camera, allowing for later viewings. The testing apparatus was cleaned thoroughly between each test with 95% ethanol to eliminate olfactory stimuli from other animals. During the test the following measures were recorded live: number of self-grooming bouts, number of rearings and leanings (lifting forepaws off the ground), and number of head dips (lowering head below the plane of the open arm). The following measures were recorded from viewing the video footage with the assistance of the behavioral analysis software *Stopwatch* (courtesy David Brown, Center for Behavioral Neuroscience, Emory University): number of open arm entries, number of closed arm entries, time spent in the open arm, and time spent in the closed arm. In ambiguous moments, when the rodent was straddling two arms, location was determined by head and forepaw position. For instance, if the mouse was positioned in the

middle of the apparatus, with its forepaws in the closed arm and its hind legs in the open arm, this occasion was scored as time spent in the closed arm. The point of intersection between arms was scored as neutral (i.e., neither closed nor open). More time spent exploring the open arms and/or an increase in the number of times the rodent enters the open arms is believed to correspond with reduced anxiety. Rearing, head dips and grooming are proposed to be indicative of exploratory behavior and reduced anxiety.

Open Field Test

Two days after the EPM, the mice were videotaped in an open field apparatus. The open field test is another standard test for anxiety-like behavior, as a rodent's natural tendency is to walk alongside the walls of the apparatus when anxious. The amount of time spent in the center of the apparatus is thus inversely correlated with anxiety levels. The open field apparatus is a 61 cm by 52 cm chamber with 31 cm high walls. Four lines are drawn on the floor of the open field, dividing the space into 9 equally sized squares. The center area is defined as being more than one inch away from the wall. Mice were placed in the chamber facing a corner and allowed to ambulate freely for five minutes. During this time the following measures were recorded: latency to first line crossing, number of line crossings, time spent in the center area, time spent near the edge, number of rearings or leanings, and number of bouts of self-grooming.

Marble-Burying Test

Mice have been shown to bury non-aversive objects such as marbles, and this behavior is attenuated by low doses of anxiolytic drugs (380, 381). Marble burying can therefore be utilized as a quick and simple test for anxiety. Clean mouse cages were filled with 1¼ inch of fresh

bedding and 20 black glass marbles were placed on the surface, in an evenly spaced array of four by five. Mice were placed in the cage for thirty minutes, during which time they explored the environment and dug through the bedding. After 30 minutes mice were returned to their home cages and the total number of exposed marbles was counted. A marble was considered buried if less than 1/3 was visible when viewed from above.

Forced Swim Test

The forced-swim test was used to measure depression-like behavior, as indicated by time spent immobile (382). A large clear glass cylinder (diameter 14 cm, height 20 cm) was filled with water at 25-27º C to a 12 cm depth. Mice were placed in the water for 6 minutes and videotaped. The container was emptied between subjects, cleaned, and refilled with water at the appropriate temperature. Video footage was used to assess the time spent immobile with the assistance of the *Stopwatch* program. Immobility was defined as floating or no active movements made other than those necessary to keep the nose above the water.

Serum hormone measurements

Serum obtained before and after the restraint test was used to measure basal and stress-induced corticosterone, respectively. Serum corticosterone levels were measured using a RIA kit from MP Biomedicals (Solon, Ohio); the sensitivity and intraassay and interassay coefficients of variance were 0.50 μg/dl, 7.7%, and 10.1%, respectively.

At the end of the present study, blood was withdrawn via cardiac puncture at the time of sacrifice. Blood was centrifuged and stored frozen at -20°C until radioimmunoassay. Serum T

levels were measured using a RIA kit from MP Biomedicals; the sensitivity and intraassay and interassay coefficients of variance were 0.02 ng/ml, 11.3%, and 12.0%, respectively.

Results

Stress Responsiveness

Blood samples were taken before and after a thirty-minute restraint stress, in which mice were placed in ventilated Plexiglas restrainers. A two-way ANOVA with repeated measures revealed a significant effect of stress ($p<0.0001$), a significant effect of genotype ($p<0.01$), but no interaction ($p=0.13$) (Figure 24A). $ER\alpha^{-/AA}$ males display significantly higher corticosterone levels in response to stress compared to both $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ males (**$p<0.01$, Bonferroni post hoc test). Post-hoc analysis of basal corticosterone demonstrates that $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males display 46% and 96% higher levels than $ER\alpha^{+/+}$ males, respectively (Figure 24B). However, this difference was only significant in $ER\alpha^{-/AA}$ males ($p<0.05$); the difference between $ER\alpha^{-/-}$ and $ER\alpha^{+/+}$ males did not reach statistical significance ($p=0.06$). Interestingly, the elevation in basal corticosterone in $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males is similar to that observed in female mice (Figure 24C). These data suggest that deletion of classical ERα signaling may essentially "feminize" basal corticosterone levels, and that non-classical signaling is sufficient to increase stress responsiveness in the absence of ERα-DNA binding.

Anxiety-like Behavior

In the elevated plus maze, there was no effect of genotype on time spent in the open arms, time spent in the closed arms, number of open arm entries, number of closed arm entries, bouts of self-grooming, or leaning and rearing ($p>0.05$, one-way ANOVA) (Figure 25).

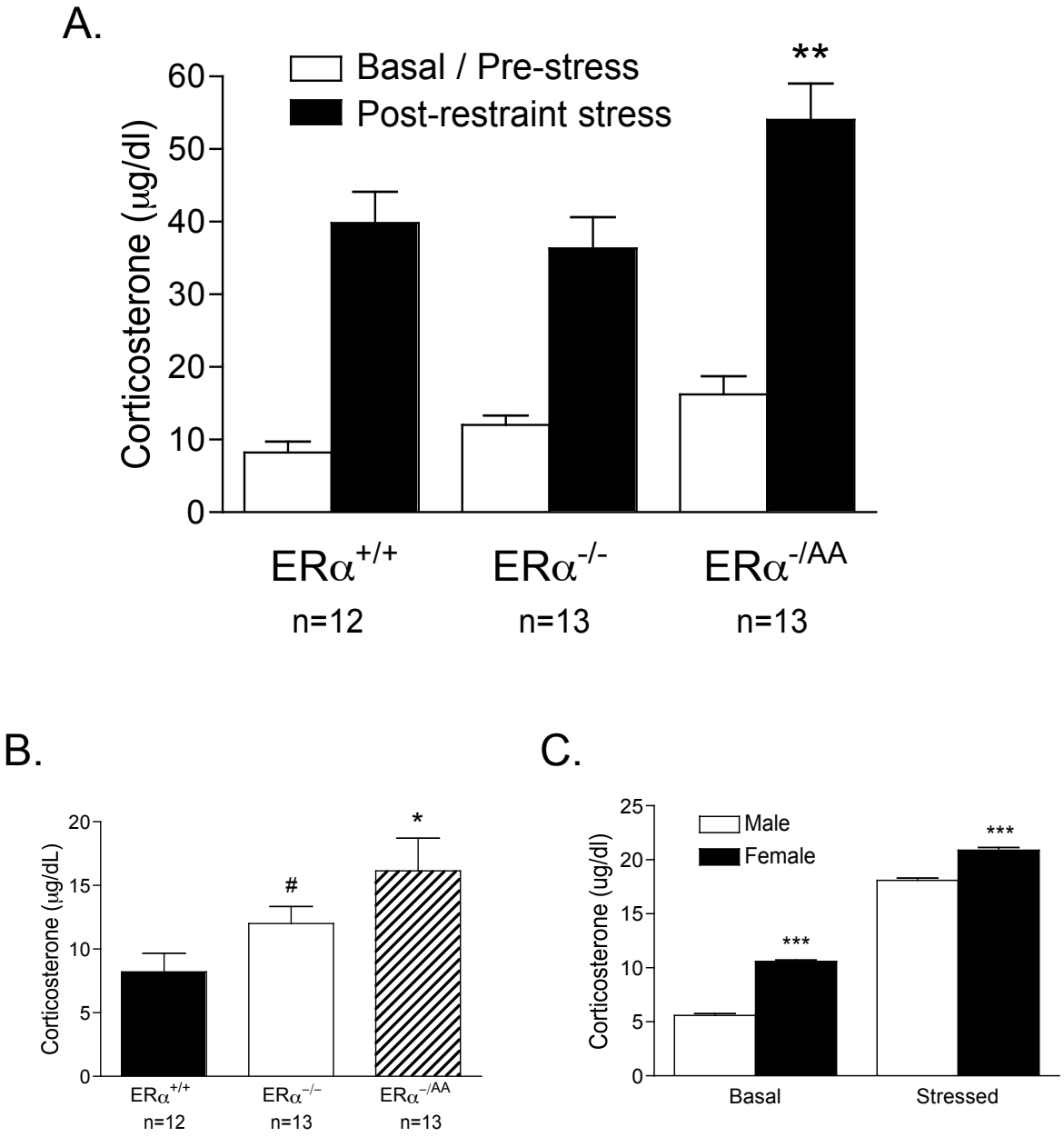


Figure 24. Serum corticosterone. (A) Blood samples were taken before and after a 30-minute restraint test. Restraint stress significantly increased serum corticosterone of all genotypes ($p<0.001$, paired t-tests). $ER\alpha^{-/AA}$ males displayed a significantly enhanced response to stress (**$p<0.01$ compared to both $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$, Bonferroni post hoc test). (B) Basal corticosterone levels are elevated in $ER\alpha^{-/-}$ and $ER\alpha^{-/AA}$ males. (*$p<0.05$ compared to $ER\alpha^{+/+}$, #$p=0.06$ compared to $ER\alpha^{+/+}$, Kruskal-Wallis with Dunn's multiple comparisons post hoc test). (C) Female mice display significantly higher basal and stress-induced corticosterone levels compared to males. (***$p<0.001$, n=698-878 per group, data courtesy of Northwestern University Neuroendocrine Core).

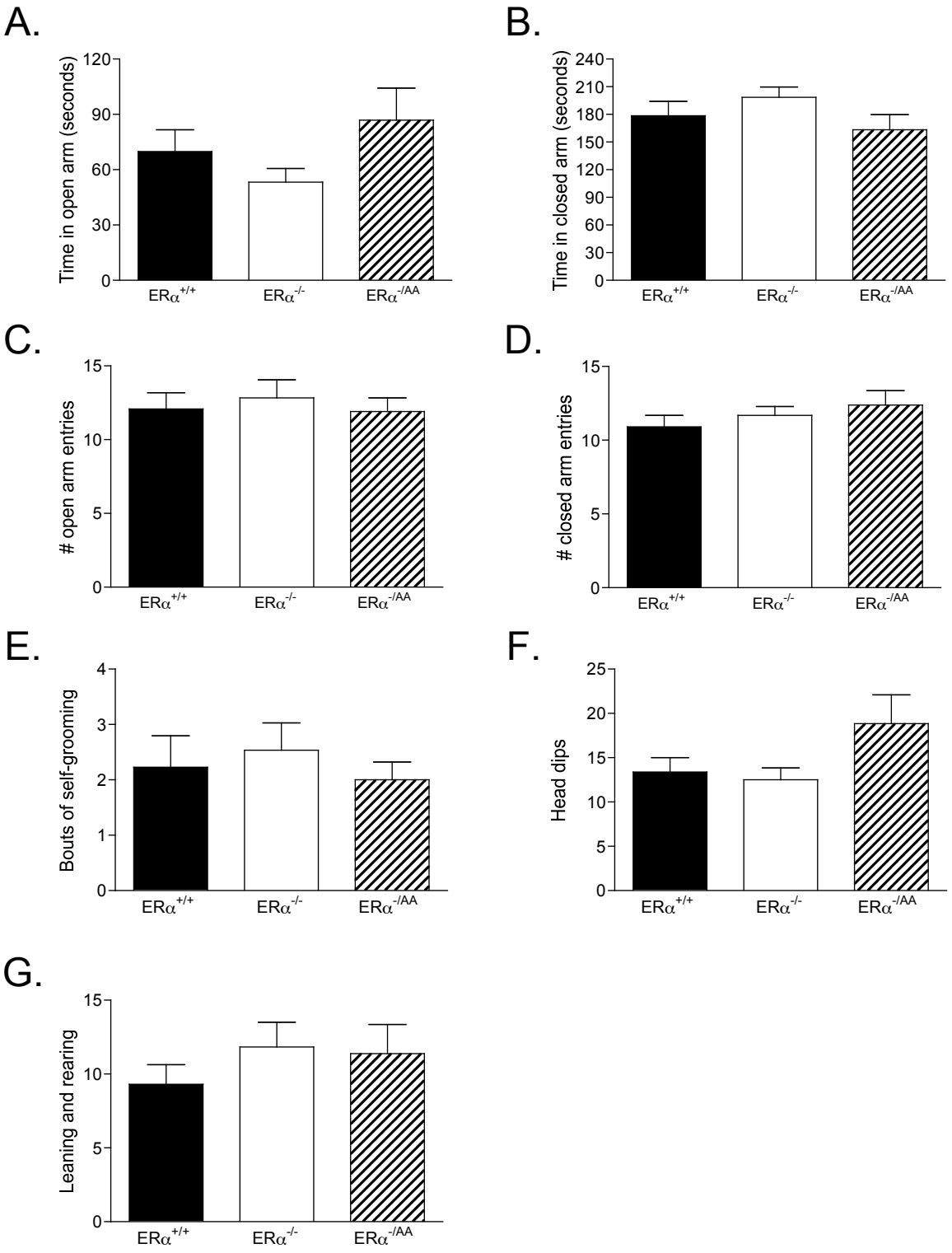


Figure 25. Anxiety-like behavior in the elevated plus maze. There was no effect of genotype on time spent in the open arm (A), time spent in the closed arm (B), number of open arm entries (C), number of closed arm entries (D), bouts of self-grooming (E), number of head dips (F), or number of leanings and rearings (G) ($p>0.05$, one-way ANOVA, n=13 per group).

In the open field test, there was no effect of genotype on time spent in the center, time spent along the edge, number of line crossings, latency to first line crossing, or leaning and rearing ($p>0.05$, one-way ANOVA) (Figure 26). However, a one-way ANOVA revealed a significant effect of genotype on bouts of self-grooming ($p<0.05$), with $ER\alpha^{-/AA}$ males displaying significantly more bouts than $ER\alpha^{+/+}$ controls ($p<0.05$, Newman Keuls post hoc test), indicating that they are less anxious in terms of this behavior.

There was also no effect of genotype on the number of marbles buried ($p>0.05$, one-way ANOVA) (Figure 27). Together these results demonstrate that ERα is not required for estrogen regulation of anxiety-like behavior in male mice. However, data from $ER\alpha^{-/AA}$ animals reveals that non-classical ERα signaling mechanisms may have anxiolytic effects in the male.

Depressive-like behavior

There was no effect of genotype on time spent immobile in the forced-swim test for depressive-like behavior ($p>0.05$, one-way ANOVA) (Figure 28), demonstrating that ERα is not required for estrogen regulation of this behavior in male mice.

Discussion

It is well established that estrogen regulates stress responsiveness, anxiety, and depression in females, but little research has focused on how estrogen affects these behaviors in males. The goal of the present study was to determine if ERα deletion disrupts these behaviors in the male, and whether ERE-dependent or ERE-independent ERα signaling mechanisms mediate effects of endogenous E_2. My findings demonstrate that ERα plays a role in regulating basal and

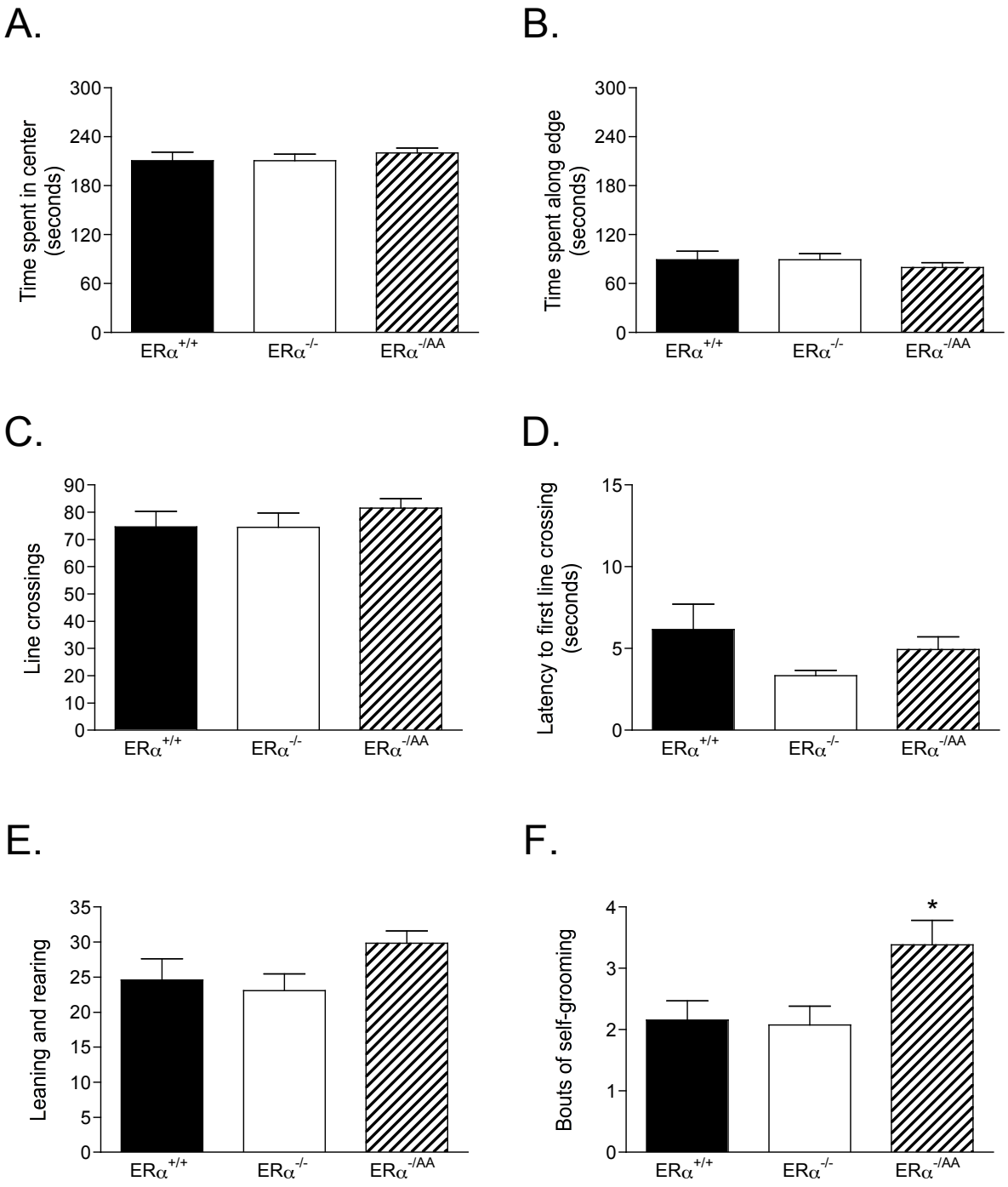


Figure 26. Anxiety-like behavior in the open field. There was no effect of genotype on time spent in the center (A), time spent along the edge (B), number of line crossings (C), latency to first line crossing (D), or number of leanings and rearings (E). There was a signifcant effect genotype on bouts of self-grooming, with $ER\alpha^{-/AA}$ males displaying signifcantly more bouts than both $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ males (*$p<0.05$, one-way ANOVA with Newman Keuls post hoc test, n=13 per group).

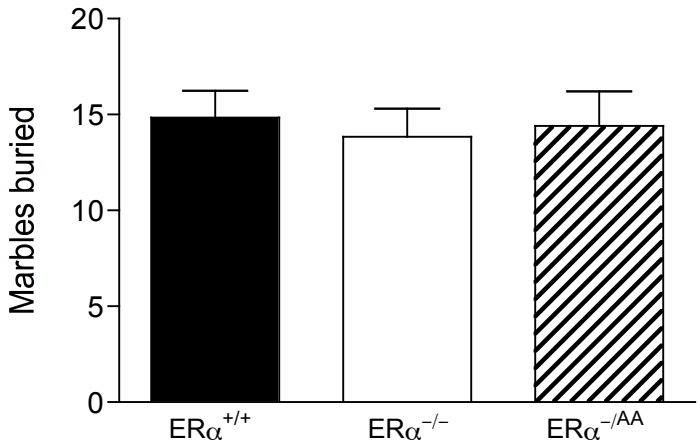


Figure 27. Anxiety-like behavior in the marble burying test. There was no effect of genotype on marble burying behavior ($p>0.05$, one-way ANOVA, n=7 per group).

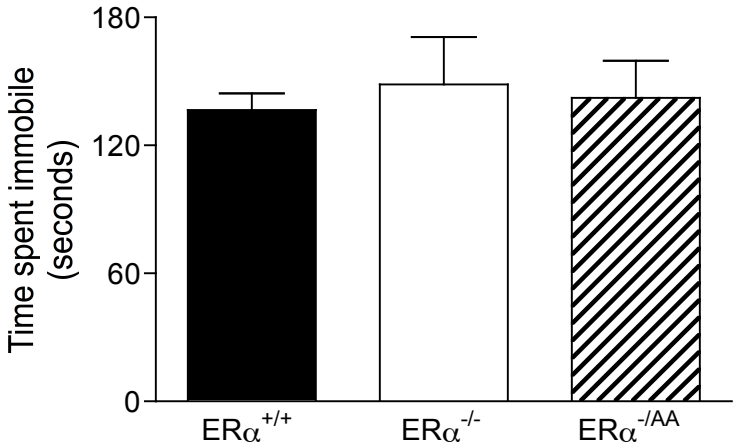


Figure 28. Depressive-like behavior in the forced swim test. There was no effect of genotype on time spent immobile ($p>0.05$, one-way ANOVA, n=7 per group).

stress-induced corticosterone secretion but does not play a role in anxiety- or depressive-like behaviors.

Basal corticosterone levels in ERα$^{-/-}$ and ERα$^{-/AA}$ males were higher than those in the ERα$^{+/+}$ control group, demonstrating that E_2 suppresses corticosterone levels via ERα. As the non-classical ERα knock-in mutation in ERα$^{-/AA}$ males was not sufficient to rescue the ERα$^{-/-}$ phenotype, these data further demonstrate that classical, ERE-dependent mechanisms are necessary for the suppression of basal corticosterone levels by E_2. Interestingly, the elevated basal corticosterone levels observed in ERα$^{-/-}$ and ERα$^{-/AA}$ males are strikingly similar to those observed in female mice, suggesting that ERα plays a role in masculinizing basal corticosterone levels. These findings are consistent with the idea that the sexual dimorphism in corticosterone release and HPA axis activity is due, at least in part, to organizational effects of estrogen during critical periods of development (358, 360). However, as ERα is deleted throughout development and adulthood, the observed differences in basal corticosterone may also reflect activational effects of E_2 via ERα. Future studies using ERα specific agonists or antagonists or antisense oligonucleotides in the adult mouse may be helpful in determining if E_2's suppressive effects on basal corticosterone are organizational or activational.

Interestingly, ERα$^{-/AA}$ males displayed a more robust response to an acute restraint stress than both ERα$^{+/+}$ and ERα$^{-/-}$ males. These data suggest that non-classical ERα mechanisms may mediate E_2 stimulation of stress responsiveness, but only in the absence of classical, ERE-dependent pathways. Previous studies have demonstrated that E_2 and the selective ERα agonists moxestrol and PPT enhance HPA reactivity to restraint in male rats, and E_2 and PPT elicit significantly enhanced restraint-induced c-fos mRNA expression in the PVN (383). Whether the

enhanced stress responsiveness in ERα$^{-/AA}$ males is due to organizational or activational effects of E_2 through ERE-independent ERα signaling pathways remains to be determined.

Future studies will be necessary to determine the exact mechanisms underlying the increase in serum corticosterone levels. ERα deletion may have a direct effect on corticosterone production from the adrenal gland, or indirect effects at the level of the hypothalamus and/or pituitary. There are a number of targets within the HPA axis that may be affected by ERα deletion, including corticotropin releasing hormone (CRH), CRH receptors (CRH-R1 or CRH-R2), adrenocorticotropic hormone (ACTH), CRH binding protein (CRH-BP), corticosteroid binding globulin (CBG), glucocorticoid receptors (GR), and steroidogenic enzymes involved in corticosterone biosynthesis. On-going studies in my laboratory are specifically examining expression of CRH in the hypothalamus, based on evidence that ERα colocalizes with CRH in the PVN (384) and that the CRH gene promoter contains an ERE (385). CRH-BP is also of particular interest, as estrogen enhances its transcription via ERα and consequently inhibits CRH and down-regulates the HPA axis (386). Moreover, CRH-BP lacks palindromic EREs in its promoter and appears to be regulated by E_2 via non-classical, ERE-independent pathways (387). Future studies may wish to additionally examine corticosterone biosynthesis in the adrenal gland, as I have demonstrated in Chapter IV that non-classical ERα signaling mediates estrogen inhibition of testosterone biosynthesis.

Results from the elevated plus maze, open field, and marble-burying tests indicate that ERα does not play a role in the expression of anxiety-like behavior in male mice. These findings and are consistent with those of Krezel and colleagues, which demonstrate that anxiety-like behavior was not different between ERαKO and wild-type males (370), but are in contrast to others that report increased or decreased anxiety in ERαKO males (180, 181). However, recent

research demonstrates that the ERα-specific agonist PPT increased anxiety in adult rats of both sexes, thus perhaps a role for ERα cannot be ruled out entirely (369). It is likely that ERβ is sufficient to mediate estrogen's effects on anxiety in ERαKO mice. Indeed, several studies have suggested that estrogen regulation of anxiety is mediated primarily through ERβ. For example, male and female ERβKO mice have been shown to display significantly increased anxiety (370). It is important to note, however, that $ER\alpha^{-/AA}$ males displayed significantly more self-grooming behavior in the open field test, which may indicate that they are less anxious. Future studies examining the role of non-classical ERα signaling in the regulation of anxiety are warranted.

The present study is the first to examine depressive-like behavior in ERαKO mice and indicates that ERα does not mediate E_2 action in the male. This is consistent with findings that ERα agonists are ineffective in reducing time spent immobile in the forced swim test (388). E_2 exerts anti-depressant-like effects in mice in the forced-swim test (389), and these effects appear to be mediated by ERβ. For example, ERβKO mice do not respond to the effects of anti-depressant effect of E_2 (390) and the ERβ-specific agonist DPN acts as an anti-depressant in rats (388). Furthermore, ERβ antisense oligonucleotides counteracted the effects of E_2 in the forced-swim test (391).

As ERα deletion had an effect on stress but not anxiety or depression, these findings demonstrate that estrogen regulation of stress and anxiety is dissociated. Evidence suggests that the HPA axis and limbic systems themselves are dissociated (392-394). While connections between these systems exist and factors such as CRH have been implicated in both stress and anxiety, stress and glucocorticoids do not necessarily promote anxiety (392).

In summary, this is the first study to examine stress responsiveness and depressive-like behavior in ERαKO male mice. The present findings demonstrate that classical, ERE-dependent signaling through ERα may play a role in masculinizing normal basal corticosterone levels in male mice. Additionally, stress responsiveness appears to be enhanced by E_2 signaling through non-classical, ERE-independent ERα pathways. In contrast, ERα does not appear to play a role in anxiety- or depressive-like behavior in male mice, suggesting that E_2 regulation of these behaviors may occur via ERβ.

APPENDIX II: A MICROARRAY ANALYSIS OF THE EFFECTS OF PRENATAL TESTOSTERONE EXPOSURE ON GENE EXPRESSION IN THE PREOPTIC AREA OF THE HYPOTHALAMUS

Abstract

Testosterone mediates sexual differentiation of brain structure and function; however, it is not clear what gene targets may contribute to T's organizational effects. The goal of the present study was to determine what genes in the preoptic area of the hypothalamus are altered by prenatal testosterone exposure. Male rats, which are exposed to endogenous testosterone from the fetal testis (control male, CM), and female rats exposed to T treatments on embryonic days 16-19 (T-treated females, TF) were compared to oil vehicle-treated control females (CF). High-density oligonucleotide microarray analysis revealed that only four genes were down-regulated and 39 genes were up-regulated in the CM group compared to the CF group. All 39 genes up-regulated in the CM group were also up-regulated in the TF group; these included cyclin D2, lin-7/Veli1/MALS1, Kras2, Marta1, Mapt (tau), tropomodulin 2, p21-activated kinase 3 (Pak3), Capon, and Tcf4/Tcf7l2. An additional 176 genes were up-regulated and five genes were down-regulated in the TF group compared to the CF group. While future quantitative analyses will be required to confirm these results, the data generated from this microarray analysis provide a foundation for the functional evaluation of genes involved in the organizational effects of androgens.

Introduction

Although genetic mechanisms are responsible for initiating gonadal differentiation, gonadal hormones are responsible for causing sexual differentiation of nongonadal tissues, including the brain. Sexual differentiation includes structural differences in the brain, as well as differences in gonadotropin secretion patterns and behaviors in adulthood. Testicular testosterone secreted during critical periods of pre- and perinatal development is necessary to masculinize and defeminize the male brain from the inherent feminization program. Males castrated shortly after birth will exhibit female-typical brain anatomy and behaviors, and females exposed to T prenatally exhibit male-typical brain anatomy and behaviors (2).

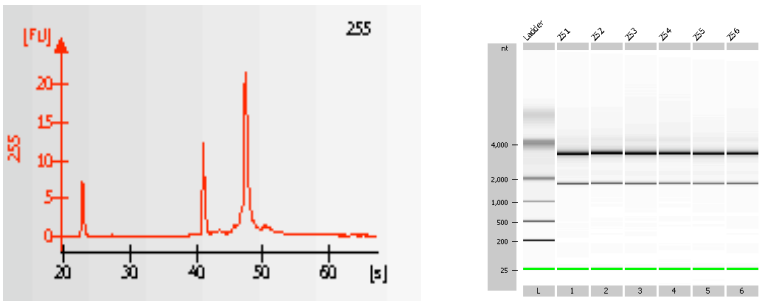
Despite the apparent role of T in mediating sexual differentiation of brain structure and function, it is not known what target gene transcripts are affected by T stimulation to contribute to the organizational effects of androgens. Microarray technology provides an efficient approach to discovering large numbers of differentially expressed mRNA transcripts as a result of early androgen exposure. I have chosen to explore gene expression in the preoptic area of the hypothalamus (POA) as it is a major target of androgen action, indicated by a high density of neurons that concentrate androgens (39). Furthermore, female rodents neonatally exposed to T exhibit masculinized sexually dimorphic nuclei of the preoptic area (SDN-POA) and display male-typical sexual behavior, which is also attributed to this region (2). Comparisons were made between vehicle-treated females and two androgen-exposed groups: vehicle-treated males and T-treated females.

Materials and Methods

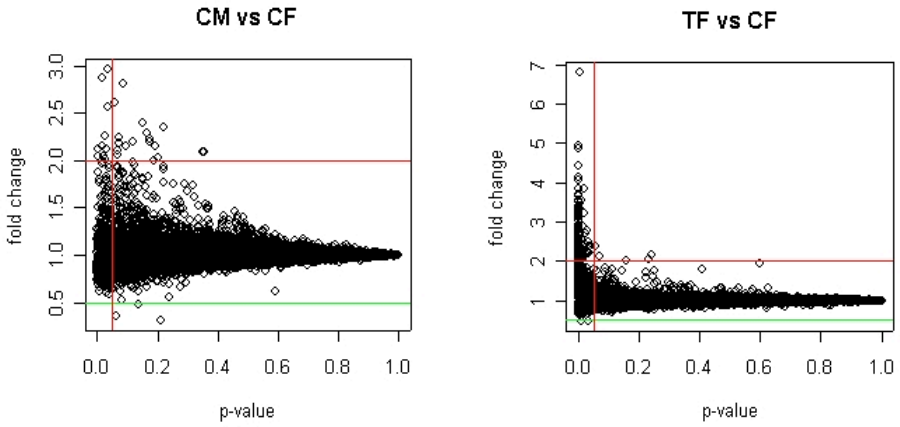
All animal and experimental procedures were conducted in accordance with the policies of Northwestern University's Animal Care and Use Committee. All rats were kept on a 14:10 light-dark cycle with lights on at 5:00 am and received food and water *ad libitum*. Timed-pregnant female Sprague-Dawley rats (180g – 300g; Charles River Laboratories, Inc., Wilmington, MA) were treated on embryonic days 16-19 of pregnancy with daily s.c. injections of either testosterone (T) or oil vehicle control. Because T has a relatively short half-life, it can be assumed that it acts specifically on these gestational days (333). T was dissolved in a 1:4 solution of benzyl benzoate: sesame oil and administered in 5 mg doses. Six intact male pups from the vehicle-treated group and eight intact female pups from the vehicle-treated and T-treated groups were used in the experiment.

Animals were anesthetized with halothane and euthanized by decapitation at postnatal day 14, at which point the rodent brain is sexually differentiated and gonadal steroid hormone levels are undetectable in both sexes. The POA was rapidly dissected, placed in ice-cold RNAlater (Ambion, Austin, TX) and stored frozen at –80˚C until RNA extraction. For isolation of RNA, tissues were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA) using a Polytron homogenizer (Brinkman Kinematica, Westbury, NY). RNA quality was confirmed for each individual sample by sharp and clean electropherogram peaks with no fragmentation peaks, and a 28S to 18S ribosomal ratio of 1.4 or greater (Figure 29). RNA from two animals (same sex, same treatment) was then combined and the RNA quality of combined samples was analyzed again. Thus, each sample is really an average of two animals; there were three replicate chips for control males, four replicate chips for control females, and four replicate chips for T-treated females.

A.



B.



C.

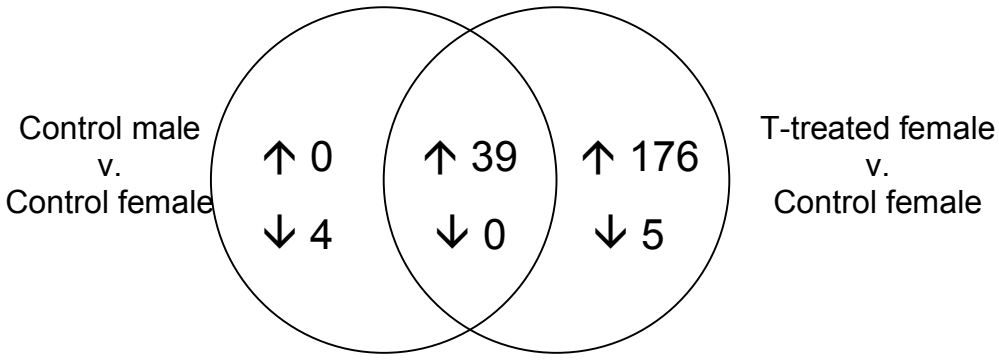


Figure 29. Microarray analysis of gene expression in the POA. (A) Representative electropherogram demonstrating sharp, clean peaks and 28S to 18S ribosomal ratio of >1.4, indicating high quality RNA. Gel shows clean bands. (B) Scatter plot of genes expressed in control male group (CM) compared to control female group (CF) and T-treated female group (TF) compared to CF group. Fold change threshold was set at 1.5 in either direction (not 2, as indicated in the image) and p value at 0.05. (C) Number of genes up- and down-regulated in each group. The intersection of the diagram represents genes that were up-regulated in both CM and TF groups compared to the CF group.

The following steps were carried out at the Center for Genetic Medicine Microarray Core Facility (Northwestern University, Chicago, IL): Integrity of total RNA samples was assessed qualitatively on an Agilent 2100 Bioanalyzer (Agilent Technologies). At least 5µg of total RNA was converted to biotinylated cRNA, according to the Affymetrix protocol for probe preparation. GeneChip Rat Expression Set 230A, a high-density oligonucleotide microarray, representing 15,923 rat genes or expressed sequence tag (EST) sequences, was purchased from Affymetrix. The biotinylated cRNA from each tissue sample was hybridized to its own GeneChip according to the Affymetrix protocol.

Statistical analyses of microarray results were performed by Dr. Lihua (Julie) Zhu and Dr. Gongxin Yu at the Northwestern University Bioinformatics Core. The Robust Multi-Array Average (RMA) package from Bioconductor was used to perform background correction for non-specific binding and for quantile normalization to unify perfect match intensity distribution across all chips. Robust probe-set summary of the log-transformed probe-level data was used in subsequent analyses and plotting. One-way ANOVA using lm in R was performed to test the effect of group. Unpaired t-tests were performed to test the difference between CF and CM, and between CF and TF, where there is a significant group effect. Results are reported as the fold change over the control-treated female group. A gene in the T-exposed groups was considered induced or repressed if the normalized average difference value was above the background value of the GeneChip and the fold change exceeded 1.5 in either direction. Significance was set at $p<0.05$ (Figure 29).

Results

When the control male group was compared to the control female group, only four genes were down-regulated and 39 genes were up-regulated (Figure 29). All 39 genes up-regulated in the control male group were also up-regulated in the T-treated female group. An additional 176 genes were up-regulated in the T-treated female group compared to the control female group and five genes were down-regulated (Figure 29). The genes up-regulated in both male and T-treated female groups and their fold change in expression over control females are listed in Table 1. The genes that are up-regulated only in the T-treated female group are listed in Table 2. The genes that are down-regulated in the control male group and in the T-treated female group are listed in Tables 3 and 4, respectively. Of note, several of the mRNAs identified here do not correspond to currently known genes, but may be searched periodically on the Affymetrix web page by probe number. As several of these unknown genes were highly up- or down-regulated, future investigation into these genes is warranted.

Discussion

The data generated from this microarray analysis is only a starting point for further functional evaluation of genes involved in the organizational effects of androgens. Quantitative follow up experiments, such as *in situ* hybridization and RT-PCR will be crucial for confirming apparent positive results. Future experiments will also explore the expression of candidate genes in additional brain regions with these more efficient approaches. It is important to note that in such a highly regulated system as the CNS, relatively slight changes in transcript levels (e.g. 1.5-fold) may be functionally significant. In fact, a small change indicated on the microarray may actually reflect a large change in a small population of neurons within the dissected POA. Thus,

with *in situ* hybridization in particular, candidate genes identified with the microarray can be localized to specific nuclei within the POA. Additional studies may wish to determine if the observed changes in gene expression at postnatal day 14 persist into adulthood.

A brief description of each of the known genes that were up-regulated in both male and T-treated female groups is given below.

Cyclin D2 (Ccnd2)

Cyclins are essential in the eukaryotic cell cycle, and function at the check point of G1 to S phase transition (395, 396). Embryonic rat brain strongly expresses the cyclin D2 gene, but its expression is dramatically repressed in matured brain (397). However, nuclear localization of cyclin D has been described in differentiated neurons in postnatal and adult stages, suggesting a physiological function beyond cell cycle regulation (398). Cyclin D2 has been shown to play a critical role in adult neurogenesis, as cyclin D2 is the only cyclin expressed in dividing cells derived from neuronal precursors present in the adult hippocampus, and *Ccnd2* mutation completely abolishes proliferation of neuronal precursors in the adult brain (399).

Lin-7-Ba (Veli1)

LIN-7 is a small protein containing little more than a single PSD-95/discs large/zona occludens-1 (PDZ) domain (400). PDZ proteins mediate receptor localization at synapses and other cell-cell junctions (401). LIN-7 is one of three PDZ-containing proteins present in epithelial cells that mediate basolateral localization of a receptor tyrosine kinase essential for vulval development in *C. elegans* (400). Mammalian homologs of LIN-7, MALS-1, -2, and -3 (for mammalian LIN-seven protein), are selectively expressed in specific neuronal populations

and are enriched in postsynaptic density (PSD) fractions (401). MALS proteins cluster together with PSD-95 and NMDA type glutamate receptors in cultured hippocampal neurons, and may regulate recruitment of neurotransmitter receptors to the PSD (401). Velis (vertebrate homologs of LIN-7) are identical to the MALS (401, 402). MALS expression is first detectable at embryonic day 13, but highest expression levels are found in the adult, suggesting that MALS may regulate aspects of synaptic plasticity in the adult brain (401).

Kirsten rat sarcoma viral oncogene homologue 2 (Kras2)

Kras2 is a member of the Ras gene family that is primarily involved in cell division. KRAS binds GDP and GTP and possesses GTPase activity. RAS proteins have been shown to influence proliferation, differentiation, transformation, and apoptosis. As an oncogene, mutations in Kras can cause cells to become cancerous; Kras is one of the most common oncogenes associated with human neoplasms (403, 404). Viral infection of Kras into a neuronal cell line leads to neuronal differentiating, neurite outgrowth, and enhances neuronal survival (405).

MAP2 RNA trans-acting protein MARTA1 (Marta1)

Microtubule-associated protein 2 (MAP2) regulates the stability of the dendritic cytoskeleton. MARTA1 is an RNA trans-acting protein that exhibits specific high-affinity binding to a dendritic targeting element in the 3' untranslated region of MAP2 and may therefore play a role in nucleocytoplasmic mRNA targeting (406). Rat MARTA1 is the orthologue of the human RNA-binding protein KSRP.

Microtubule-associated protein tau (Mapt)

Mapt (or tau) plays a major role in promoting microtubule assembly and stabilization, and in maintaining neuronal morphology. It may also participate in the regulation of intracellular signal transduction, and the development and viability of neurons. Gene mutations, aberrant mRNA splicing, and posttranslational modifications are associated with a number of neurodegenerative disorders (407). Interestingly, abnormal hyperphosphorylation of Mapt is associated with Alzheimer's disease, and hyperphosporylation is prevented by androgens. Thus, androgens may exert a neuroprotective effect and explain why Alzheimer's is more prevalent in women than men (408).

Tropomodulin 2 (Tmod2)

Tropomodulin is a cytoskeletal protein that caps the pointed ends of actin filaments and inhibits tropomyosin binding to actin (409). Tmod2 is highly expressed in neuronal tissue and may play a role in axonal and neurite outgrowth and synaptic plasticity. Mice deficient for Tmod2 are hyperactive and display reduced sensorimotor gating and impaired learning and memory (410).

p21-activated kinase 3 (Pak3)

The p21 activated kinases (PAKs) are serine/threonine kinase that can be activated by small GTPases and are involved in the regulation of cytoskeleton reorganization, dendrite morphogenesis, synaptogenesis, neuroplasticity, cellular proliferation, cell morphology, apoptosis, and cell cycle progression (411, 412). Pak3 is expressed exclusively in the central nervous system, with high mRNA expression in the spinal cord, thalamus, hypothalamus,

midbrain and pons medulla (413, 414). Mutations in the human Pak3 gene are responsible for X-linked mental retardation (415, 416). Notably, PAK1 and PAK3 have been demonstrated to phosphorylate synapsin I, which complexes with nNOS and CAPON (discussed below). On-going studies in my laboratory are examining the role of PAK1 in the timing of the E-induced LH surge in female rats (Zhen Zhao, unpublished observations).

C-terminal PDZ domain ligand of neuronal nitric oxide synthase (Capon)

Neuronal nitric oxide synthase (nNOS) is the enzyme that generates nitric oxide, which has a number of roles in neural tissue including regulating neurotransmitter release. nNOS is linked to postsynaptic densities (PSD) by the cytoskeletal protein PSD-95 (417), which places it near other PSD-95 ligands, such as the NR2 subunit of the NMDA receptor (418). Accordingly, NMDA receptor stimulation efficiently activates nNOS (419). The PDZ domain of nNOS also interacts with the PDZ domain of the adapter protein CAPON, which may influence nNOS by regulating its ability to associate with PSD95/NMDA receptor complexes (420). Interestingly, CAPON has been identified as a positional candidate for the schizophrenia-susceptibility locus through linkage disequilibrium mapping (421-423).

Transcription factor 4 (Tcf4)

Tcf4 is a member of the Tcf/Lef family of transcription factors that forms a complex with beta-catenin and mediates Wnt signaling by transactivating target genes. Tcf4 expression appears to be restricted to the developing midbrain and overlaps with the expression pattern of members of the Wnt family (424-426). The Wnt signaling pathway is critical for normal embryogenesis, cell proliferation, cell motility, and cell fate determination. Tcf4 is also known as transcription

factor 7-like 2 (TCF7L2) and has been identified as a type 2 diabetes-susceptibility gene; variants of this gene are more strongly associated with type 2 diabetes risk than any other gene identified to date (427).

Although numerous cellular features have been found to be sexually dimorphic, such as cell size, neural connectivity, neurotransmitter content, and receptor expression, the mechanisms underlying the organizational effects of androgens are not completely understood (428). Since androgen exposure has been shown to increase cell density in the SDN-POA, it is perhaps not surprising that many of the T-induced genes identified in this microarray are involved in neurogenesis, neuronal migration or neuronal survival. Nonetheless, the present study is the first large-scale evaluation of the effects prenatal T-exposure on gene expression in the rat preoptic area and provides a terrific starting point for further functional evaluation of genes involved in the organizational effects of androgens.

Table 1. Genes up-regulated in the POA of both male and prenatally T-treated female rats compared to control female rats on PND 14

Probe	Gene Name	Gene Symbol	One-way ANOVA p value	TF v CF p value	TF v CF Fold	CM v CF p value	CM v CF Fold
1375650_at	Similar to bromodomain-containing protein BRD4 short variant	---	0.0021	1.9E-05	4.45	0.0348	2.97
1370810_at	Cyclin D2	Ccnd2	0.0001	0.0001	4.06	0.0175	2.87
1373804_at	Similar to forkhead-related transcription factor 1A	---	0.0083	0.0085	2.12	0.0316	2.12
1387071_a_at	microtubule-associated protein tau	Mapt	0.0003	0.0001	2.21	0.0026	2.04
1370108_a_at	lin-7-Ba	Veli1	0.0045	0.0002	2.37	0.0384	1.93
1375426_a_at	MAP2 RNA trans-acting protein MARTA1	Marta1	0.0026	0.0003	2.16	0.0311	1.78
1370035_at	Kirsten rat sarcoma viral oncogene homologue 2 (active)	Kras2	0.0009	4.8E-05	2.24	0.0318	1.68
1375424_at	Similar to actin-related protein 2	---	0.0032	0.0006	1.85	0.0250	1.63
1370438_at	C-terminal PDZ domain ligand of neuronal nitric oxide synthas	Capon	0.0008	0.0004	1.60	0.0170	1.63
1368902_at	p21 (CDKN1A)-activated kinase 3	Pak3	0.0002	0.0001	1.78	0.0155	1.56
1368841_at	transcription factor 4	Tcf4	0.0026	0.0002	1.81	0.0417	1.54
1369541_at	tropomodulin 2	Tmod2	0.0026	0.0017	2.48	0.0049	1.51
1377651_at	---	---	0.0027	0.0009	3.83	0.0353	2.56
1385889_at	---	---	0.0001	0.0011	3.25	0.0248	2.26
1375215_x_at	---	---	0.0022	0.0004	2.80	0.0152	2.15
1389974_at	---	---	1.4E-05	8.6E-05	2.89	0.0045	2.12
1375212_at	---	---	0.0004	0.0003	2.62	0.0210	2.04
1373067_at	---	---	0.0002	3E-05	2.85	0.0276	2.02
1392246_at	---	---	0.0004	0.0015	3.33	0.0231	1.98
1376138_at	---	---	0.0009	0.0012	2.70	0.0173	1.96
1375532_at	---	---	0.0004	0.0002	3.36	0.0415	1.95
1377823_at	---	---	0.0006	0.0042	1.84	0.0051	1.88
1393505_x_at	Similar to RIKEN cDNA B230380D07	---	0.0046	0.0007	1.98	0.0313	1.83
1383162_at	---	---	0.0016	0.0002	2.26	0.0284	1.80
1389905_at	---	---	0.0033	0.0003	2.61	0.0070	1.80
1376654_at	---	---	0.0074	0.0008	1.87	0.0085	1.76
1377807_a_at	---	---	0.0003	2.2E-05	2.54	0.0364	1.76
1390852_x_at	---	---	0.0007	3.4E-05	2.19	0.0237	1.73
1375538_at	---	---	0.0010	0.0013	2.18	0.0352	1.73
1398421_at	---	---	0.0118	0.0015	1.70	0.0497	1.71
1375724_at	---	---	0.0094	0.0013	1.96	0.0422	1.67
1375280_at	Similar to 5830417C01Rik protein	---	0.0021	0.0034	1.91	0.0488	1.66
1372775_at	---	---	0.0036	0.0029	1.63	0.0131	1.61
1375237_at	---	---	0.0044	0.0006	2.22	0.0337	1.61
1377808_at	---	---	0.0013	7.9E-05	2.05	0.0250	1.58
1394234_x_at	---	---	0.0013	0.0014	1.79	0.0121	1.53
1390443_at	---	---	0.0003	0.0004	1.76	0.0028	1.51
1377445_at	---	---	0.0232	0.0104	1.88	0.0434	1.51
1375714_at	Similar to mKIAA1225 protein	---	0.0279	0.0016	1.63	0.0200	1.50

Table 2. Genes up-regulated in the POA of prenatally T-treated female rats compared to control female rats on PND 14

Probe	Gene Name	Gene Symbol	TF v CF p value	TF v CF Fold
1370950_at	ER transmembrane protein Dri 42	Ppap2b	0.0002	4.85
1390048_at	Similar to splicing coactivator subunit SRm300; RNA binding protein; AT-rich element binding fact	---	0.0017	4.08
1368405_at	v-ral simian leukemia viral oncogene homolog A (ras related)	Rala	0.0002	3.70
1368958_at	protein kinase C and casein kinase substrate in neurons 1	Pacsin1	0.0018	3.56
1370946_at	Nuclear factor I/X	Nfix	0.0035	3.32
1368778_at	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	Slc6a6	0.0003	3.21
1375703_at	Similar to MLL5	---	0.0005	3.18
1388167_at	nuclear factor I/B	Nfib	0.0005	3.14
1370512_at	androgen receptor-related apoptosis-associated protein CBL27	Cbl27	0.0015	2.95
1387929_at	PMF32 protein	Pmf31	0.0001	2.88
1389116_at	myotubularin related protein 9	Mtmr9	0.0004	2.82
1369654_at	protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	0.0019	2.77
1373863_at	Similar to Traf2 and NCK interacting kinase, splice variant 4	---	0.0004	2.68
1392890_at	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	Pafah1b1	0.0041	2.66
1375469_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, membe	Smarca4	0.0040	2.65
1369215_a_at	carboxypeptidase D	Cpd	0.0019	2.60
1389840_at	Splicing factor 3b, subunit 1, 155kD	Sf3b1	0.0002	2.58
1383053_x_at	Similar to zinc finger protein 91 isoform 1	---	0.0005	2.57
1376917_at	Similar to zinc-finger protein	---	0.0008	2.54
1393418_at	tropomodulin 2	Tmod2	0.0012	2.50
1387659_at	guanine deaminase	Gda	0.0064	2.39
1368979_at	huntingtin-associated protein interacting protein (duo)	Hapip	0.0006	2.37
1373494_at	Similar to breakpoint cluster region protein	---	0.0013	2.36
1383013_at	Similar to Krueppel-like factor 13 (Transcription factor BTEB3) (RFLAT-1) (Transcription factor N5	---	0.0008	2.34
1368899_at	bone morphogenetic protein receptor, type 1A	Bmpr1a	0.0293	2.25
1368255_at	neurotrimin	RNU16845	0.0001	2.25
1370705_at	zinc finger protein HIT-4	Hit4	0.0060	2.20
1368440_at	solute carrier family 3, member 1	Slc3a1	0.0042	2.19
1398251_a_at	calcium/calmodulin-dependent protein kinase II beta subunit	Camk2b	0.0024	2.19
1370830_at	epidermal growth factor receptor	Egfr	0.0033	2.15
1387146_a_at	endothelin receptor type B	Ednrb	0.0041	2.15
1383075_at	cyclin D1	Ccnd1	0.0004	2.15
1389340_at	Similar to zinc finger protein TZF-L	---	0.0005	2.12
1375362_at	Similar to RIKEN cDNA 2010106G01	---	0.0019	2.09
1387572_at	pleckstrin and Sec7 domain containing	Psd	0.0042	2.09
1370048_at	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Edg2	0.0157	2.05
1370267_at	glycogen synthase kinase 3 beta	Gsk3b	0.0003	2.05
1374085_at	Similar to Max dimerization protein 4	---	0.0006	1.98
1369425_at	cadherin 13	Cdh13	0.0001	1.98

Table 2, continued

Probe	Gene Name	Gene Symbol	TF v CF p value	TF v CF Fold
1370050_at	ATPase, Ca++ transporting, plasma membrane 1	Atp2b1	0.0025	1.97
1371148_s_at	internexin, alpha	Inexa	0.0008	1.96
1375177_at	Similar to Krueppel-like factor 13 (Transcription factor BTEB3) (RFLAT-1) (Transcription factor NS	---	0.0025	1.93
1387204_at	neuronal growth regulator 1	Negr1	0.0012	1.91
1370831_at	monoglyceride lipase	Mgll	0.0013	1.91
1369993_at	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	Camk2g	0.0025	1.89
1367728_at	translin	Tsn	0.0000	1.88
1377061_at	RhoGAP involved in beta-catenin-N-cadherin and NMDA receptor signaling	RICS	0.0046	1.87
1369679_a_at	nuclear factor I/A	Nfia	0.0010	1.86
1376931_at	Similar to Hepatocellular carcinoma-associated antigen 58 homolog	---	0.0017	1.85
1368320_at	neural cell adhesion molecule 1	Ncam1	0.0005	1.84
1387703_a_at	ubiquitin specific protease 2	Usp2	0.0002	1.83
1375214_at	Similar to UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2	---	0.0037	1.82
1398262_at	phosphoribosyl pyrophosphate synthetase 2	Prps2	0.0046	1.82
1370957_at	interleukin 6 signal transducer	Il6st	0.0032	1.81
1369355_at	glutamate receptor, metabotropic 5	Grm5	0.0032	1.80
1368842_at	transcription factor 4	Tcf4	0.0010	1.79
1370935_at	CDW92 antigen	Cdw92	0.0062	1.79
1387932_at	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), membe	Slc1a1	0.0008	1.79
1374593_at	protein kinase C, epsilon	Prkce	0.0023	1.79
1390000_at	Similar to KIAA0346	---	0.0057	1.78
1370262_at	LYRIC	Lyric	0.0011	1.77
1369680_at	solute carrier family 2 (facilitated glucose transporter), member 13	Slc2a13	0.0026	1.76
1375119_at	Neural precursor cell expressed, developmentally down-regulated gene 4A	Nedd4a	0.0003	1.74
1373860_at	Similar to sox-4 protein - mouse	---	0.0000	1.74
1384610_at	Similar to RIKEN cDNA B230380D07	---	0.0027	1.73
1387289_at	amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	Apba2	0.0000	1.72
1370951_at	ER transmembrane protein Dri 42	Ppap2b	0.0048	1.72
1375719_s_at	cadherin 13	Cdh13	0.0002	1.71
1398846_at	eukaryotic translation initiation factor 5	Eif5	0.0059	1.70
1372812_at	Similar to Hypothetical protein KIAA0256	---	0.0014	1.70
1372480_at	Similar to KIAA1803 protein	---	0.0004	1.68
1369868_at	implantation-associated protein	IAG2	0.0115	1.68
1370517_at	neuronal pentraxin 1	Nptx1	0.0019	1.67
1368202_a_at	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	Dab2	0.0008	1.66
1387024_at	dual specificity phosphatase 6	Dusp6	0.0003	1.65
1368871_at	mitogen activated protein kinase kinase kinase 1	Map3k1	0.0001	1.65
1375428_at	Similar to cellular repressor of E1A-stimulated genes CREG	---	0.0408	1.65
1389918_at	Similar to palladin; CGI-151 protein	---	0.0099	1.64

Table 2, continued

Probe	Gene Name	Gene Symbol	TF v CF p value	TF v CF Fold
1376761_at	Histone deacetylase 4	Hdac4	0.0006	1.64
1375905_at	Similar to leukocyte receptor cluster (LRC) member 8	---	0.0025	1.63
1369524_at	Zic family member 1 (odd-paired homolog, Drosophila)	Zic1	0.0006	1.63
1370545_at	potassium voltage-gated channel, shaker-related subfamily, member 1	Kcna1	0.0121	1.62
1369404_a_at	neurexin 1	Nrxn1	0.0012	1.62
1388002_at	serine/threonine protein kinase TAO1	LOC286993	0.0010	1.61
1376146_at	Similar to RIKEN cDNA 2310033P09	---	0.0011	1.61
1368972_at	neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	0.0004	1.60
1369410_at	golgi SNAP receptor complex member 1	Gosr1	0.0009	1.60
1370384_a_at	prolactin receptor	Prlr	0.0305	1.60
1371481_at	Similar to POLYPOSIS LOCUS PROTEIN 1 HOMOLOG (TB2 PROTEIN HOMOLOG) (GP106)	---	0.0062	1.59
1368393_at	lymphocyte antigen 68	C1qr1	0.0014	1.58
1390502_at	Similar to RIKEN cDNA 1700025G04 gene	---	0.0038	1.58
1367979_s_at	cytochrome P450, subfamily 51	Cyp51	0.0028	1.58
1376758_at	Similar to ING1 protein	---	0.0009	1.58
1387265_at	diacylglycerol kinase, gamma	Dgkg	0.0008	1.57
1368258_at	apelin, AGTRL1 ligand	Apln	0.0073	1.57
1376263_at	Similar to 1810034B16Rik protein	---	0.0046	1.57
1369559_a_at	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	Cd47	0.0030	1.56
1377526_at	PDZ protein Mrt1	Snx27	0.0033	1.56
1371094_at	LIM homeobox protein 2	Lhx2	0.0113	1.56
1370043_at	activated leukocyte cell adhesion molecule	Alcam	0.0018	1.55
1375533_at	Similar to Hypothetical protein MGC54805	---	0.0061	1.54
1384609_a_at	Similar to RIKEN cDNA B230380D07	---	0.0009	1.53
1387961_at	opioid-binding protein/cell adhesion molecule-like	Opcml	0.0005	1.53
1375305_at	LOC362256	---	0.0075	1.53
1367823_at	tissue inhibitor of metalloproteinase 2	Timp2	0.0005	1.53
1375641_at	Similar to RIKEN cDNA 2010015J01	---	0.0016	1.51
1377300_at	Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	Dusp3	0.0012	1.51
1371202_a_at	nuclear factor I/B	Nfib	0.0017	1.51
1389868_at	---	---	0.0030	6.82
1383052_a_at	---	---	0.0001	4.94
1380433_at	---	---	0.0006	4.13
1375676_at	---	---	0.0018	3.67
1376933_at	---	---	0.0009	3.53
1383054_at	---	---	0.0006	3.28
1376419_at	---	---	0.0005	3.19
1376463_at	---	---	0.0021	3.10
1390378_at	---	---	0.0002	3.03

Table 2, continued

Probe	Gene Name	Gene Symbol	TF v CF p value	TF v CF Fold
1390592_at	---	---	0.0007	2.92
1390398_at	---	---	0.0131	2.90
AFFX-r2-Ec-bioC-5_a	---	---	0.0235	2.72
1382778_at	---	---	0.0065	2.62
1390100_s_at	---	---	0.0007	2.55
AFFX-BioDn-5_at	---	---	0.0317	2.50
1376175_at	---	---	0.0090	2.47
AFFX-r2-Ec-bioD-3_a	---	---	0.0241	2.44
AFFX-r2-Ec-bioD-5_a	---	---	0.0170	2.40
1375723_at	---	---	0.0010	2.38
AFFX-r2-Ec-bioC-3_a	---	---	0.0392	2.37
1377029_at	---	---	0.0087	2.36
AFFX-CreX-5_at	---	---	0.0116	2.34
1390506_at	---	---	0.0007	2.31
AFFX-BioC-5_at	---	---	0.0426	2.28
1375687_at	---	---	0.0000	2.25
1383577_at	---	---	0.0014	2.20
AFFX-CreX-3_at	---	---	0.0117	2.19
1390116_at	---	---	0.0009	2.10
1375707_at	---	---	0.0020	2.10
1376627_at	---	---	0.0030	2.08
1379506_at	---	---	0.0009	2.04
1393494_at	---	---	0.0024	2.03
1377151_at	---	---	0.0009	2.03
1393268_at	---	---	0.0022	2.02
1376848_at	---	---	0.0006	1.99
1399090_at	---	---	0.0004	1.95
1376885_at	---	---	0.0002	1.91
1376157_at	---	---	0.0034	1.85
1390351_at	---	---	0.0108	1.84
1385594_at	---	---	0.0009	1.84
1399100_at	---	---	0.0011	1.84
AFFX-r2-P1-cre-5_at	---	---	0.0063	1.80
1377412_at	---	---	0.0065	1.80
1392566_at	---	---	0.0016	1.78
1375916_at	---	---	0.0058	1.77
1376127_at	---	---	0.0022	1.73
1390077_at	---	---	0.0015	1.73

Table 2, continued

Probe	Gene Name	Gene Symbol	TF v CF p value	TF v CF Fold
1383161_a_at	---	---	0.0017	1.73
1381489_at	---	---	0.0042	1.71
AFFX-r2-P1-cre-3_at	---	---	0.0214	1.71
1376725_at	---	---	0.0149	1.70
1375925_at	---	---	0.0015	1.69
1390231_at	---	---	0.0009	1.69
1390722_at	---	---	0.0000	1.67
1376771_at	---	---	0.0032	1.66
1389132_at	---	---	0.0019	1.60
1376350_at	---	---	0.0112	1.59
1383331_at	---	---	0.0014	1.58
1377713_at	---	---	0.0046	1.58
1375699_at	---	---	0.0073	1.56
1376739_at	---	---	0.0115	1.56
1381968_at	---	---	0.0455	1.56
1377042_at	---	---	0.0033	1.52
1375788_at	---	---	0.0004	1.51
1379511_at	---	---	0.0463	1.51
1392746_x_at	---	---	0.0036	1.51
1389526_at	---	---	0.0039	1.51
1389104_s_at	---	---	0.0012	1.50

Table 3. Genes down-regulated in the POA of male rats compared to control female rats on PND 14

Probe	Gene Name	Gene Symbol	One-way ANOVA p value	CM v CF p value	CM v CF Fold
1374684_at	---	---	0.0033	0.0287	0.59
1375535_at	---	---	0.0022	0.0055	0.61
1376745_at	---	---	0.0123	0.0495	0.65
1369283_at	tubby homolog (mouse)	Tub	0.0323	0.0335	0.65

Table 4. Genes down-regulated in the POA of prenatally T-treated female rats compared to control female rats on PND 14

Probe	Gene Name	Gene Symbol	One-way ANOVA p value	TF v CF p value	TF v CF Fold
1372577_at	---	---	0.0198	0.0335	0.50
1374370_at	---	---	0.0127	0.0044	0.63
1374655_at	---	---	0.0111	0.0090	0.47
1376780_at	Similar to RIKEN cDNA 2610204K14	---	0.0035	0.0333	0.47
1376867_at	Similar to RIKEN cDNA 1110067D22	---	0.0003	0.0053	0.66

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Curriculum Vita, June 2008

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EDUCATION

2002-present Ph.D. candidate, Neurobiology and Physiology
Interdepartmental Biological Sciences (IBiS) Program
Northwestern University, Evanston, Illinois

1998-2002 B.S., Biology
University of Michigan, Ann Arbor, Michigan

RESEARCH EXPERIENCE

2002-present Graduate Student
Department of Neurobiology and Physiology
Northwestern University, Evanston, Illinois
Advisor: Jon E. Levine, Ph.D.
Dissertation: "Cellular and molecular mechanisms of androgen action in male reproduction"

2001-2002 Undergraduate Research Assistant
Department of Molecular and Integrative Physiology
University of Michigan Medical School, Ann Arbor, Michigan
Advisor: John A. Faulkner, Ph.D.
Research Project: "Contraction-induced injury in mammalian skeletal muscle"

TEACHING EXPERIENCE

2004-2008 Mentor for four undergraduate students' honors thesis projects

2007 Tutor Group Leader
Science & Engineering Research & Teaching Synthesis (SERTS)
Biology 101 - Biology in the Information Age
Instructor: Theresa Horton, Ph.D.

2004-2007 Tutorial Instructor
Science & Engineering Research & Teaching Synthesis (SERTS)
Biology 101 - Biology in the Information Age
Instructor: Theresa H. Horton, Ph.D.
Designed and conducted small group tutorial sessions for non-major undergraduate students

2005 Tutorial Instructor
Science & Engineering Research & Teaching Synthesis (SERTS)
Anthropology 213 - Human Origins
Instructor: J. Josh Snodgrass, Ph.D.

2005 Teaching Assistant
Biological Science 385 – Vertebrate Endocrinology
Instructor: Jon E. Levine, Ph.D.
Answered questions and advised students during office hours, graded exams, and managed course website.

2003 Teaching Assistant
Biological Science 210-1 – Genetics and Evolution
Instructors: Robert Holmgren, Ph.D., Joseph Walsh, Ph.D.
Supervised two laboratory sections and led two discussion sections, designed quizzes, graded exams and lab reports, and advised students.

AWARDS AND FELLOWSHIPS

2008 Northwestern University Graduate School Travel Grant

2008 Interdepartmental Biological Sciences (IBiS) Travel Award

2006 International Neuroendocrine Federation Young Investigator Travel Award

2005-2008 National Institute of Health Ruth L. Kirschstein National Research Service Award, Northwestern University Reproductive Biology Training Grant

2005, 2007 Constance Campbell Memorial Research Award, Oral division, 2nd place
Northwestern University Center for Reproductive Science Annual Minisymposium on Reproductive Biology

2004 Constance Campbell Memorial Research Award, Poster division, 2nd place
Northwestern University Center for Reproductive Science 25th Annual Minisymposium on Reproductive Biology

PROFESSIONAL MEMBERSHIPS

American Neuroendocrine Society
Endocrine Society
Society for Behavioral Neuroendocrinology
Society for Neuroscience
Women in Endocrinology

ADDITIONAL EXPERIENCE

2006	Edited *Endocrinology* textbook (6th edition, Prentice Hall) in collaboration with research advisor, Jon Levine
2006	Chairperson for the Northwestern University Center for Reproductive Science 27th Annual Minisymposium on Reproductive Biology
2004	Interdepartmental Biological Sciences (IBiS) Program Graduate Student Recruitment Committee Member
2003-2004	IBiS Student Organization Social Co-Chair

PUBLICATIONS

McDevitt MA, Glidewell-Kenney C, Weiss J, Chambon P, Jameson JL, Levine JE. (2007). Estrogen response element-independent estrogen receptor alpha (ERa) signaling does not rescue sexual behavior but restores normal testosterone secretion in male ERa knockout mice. *Endocrinology*, 148(11):5288-94.

Foecking EM, McDevitt MA, Acosta-Martinez M, Horton TH, Levine JE. (2008). Neuroendocrine consequences of androgen excess in female rodents. *Hormones and Behavior*, 53(3):673-692. Invited review for a special issue on androgens.

McDevitt MA, Levine JE, Glidewell-Kenney C, Weiss J, Jameson JL. (2008). New insights into the classical and nonclassical actions of estrogen in the brain: Evidence from estrogen receptor knock out and knock in mice. *Molecular and Cellular Endocrinology*, *In press*. Invited review for a special issue on estrogen action in the brain.

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McDevitt MA, Glidewell-Kenney C, Capshew C, Chen BB, Ge RS, Hardy MP, Weiss J, Jameson JL, Levine JE. ERE-independent ERα signaling mediates estrogen inhibition of androgen biosynthesis in the mouse testis. *In preparation*.

Bielsky IF, Schmitz SR, McDevitt MA, Levine JE. Prenatal androgen exposure alters anxiety-like but not aggression behavior in adult male rats. *In preparation*.

CONFERENCE PRESENTATIONS

McDevitt MA, Glidewell-Kenney C, Capshew C, Chen BB, Ge RS, Hardy MP, Weiss J, Jameson JL, Levine JE. (2008). ERE-independent ERα signaling mediates estrogen inhibition of androgen biosynthesis in the mouse testis. Poster to be presented at the Endocrine Society 90th Annual Meeting, San Francisco, CA.

McDevitt MA, Glidewell-Kenney, Weiss J, Chambon P, Jameson JL, Levine JE. (2007). ERE-independent ERα signaling does not rescue sexual behavior but restores normal testosterone secretion in male ERαKO mice. Oral presentation given at the Northwestern University Center for Reproductive Science 28th Annual Minisymposium on Reproductive Biology, Evanston, IL, and poster presented at the Society for Neuroscience 37th Annual Meeting, San Diego, CA.

Chamberlin MA, Lauder AS, Thorsness RJ, Levine JE. (2006). Mounting behavior of castrated male rats is rescued by infusion of an ATP-sensitive potassium channel inhibitor in the preoptic area. Poster presented at the Society for Behavioral Neuroendocrinology 10th Annual Meeting and 6th International Congress of Neuroendocrinology, Pittsburg, PA.

Chamberlin MA, Thorsness RJ and Levine JE. (2005). Blockade of ATP-sensitive potassium channels restores male sexual behavior in the castrated rat. Oral presentation given at the Northwestern University Center for Reproductive Science 26th Annual Minisymposium on Reproductive Biology, Evanston, IL, and poster presented at the Society for Neuroscience 35th Annual Meeting, Washington, DC.

Chamberlin MA and Levine JE. (2004). Androgens inhibit ATP-sensitive potassium channel subunit mRNA expression in the male rat preoptic area and medial basal hypothalamus. Poster presented at the Northwestern University Center for Reproductive Science 25th Annual Minisymposium on Reproductive Biology, Evanston, IL, and at the Society for Neuroscience 34th Annual Meeting, San Diego, CA.