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The Roles of Aromatization and Steroid Receptor Activation in the Restoration of Sociosexual Behavior in Adult Male Rats

By

Michael E. Vagell

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1998

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ABSTRACT

The Roles of Aromatization and Steroid Receptor Activation in the Restoration of Sociosexual Behavior in Adult Male Rats

By

Michael E. Vagell

Adviser: Marilyn McGinnis, Ph.D.

This work assessed the role of aromatization and steroid receptor binding in testosterone's restoration of several sociosexual behaviors (such as copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking) in testosterone-treated gonadectomized male rats. Preventing the aromatization of testosterone to estradiol with the aromatase inhibitor fadrozole, completely blocked the restoration of copulatory behavior, but not partner preference. Moreover, treatment with estradiol alone was not sufficient to restore copulatory behavior or partner preference. The two major findings from these experiments were: (1) both androgens and estrogens were necessary for the restoration of copulatory behavior, and (2) aromatization was necessary for the restoration of copulatory behavior, but not partner preference.

Steroid hormones have been traditionally thought to act in the brain by binding to intracellular receptors, which act as ligand-inducible transcription factors. For instance, blocking androgen receptors with the antiandrogen hydroxyflutamide significantly inhibits testosterone's restoration of copulatory behavior, partner preference, 50 kHz ultrasonic vocalizations, and scent marking. This supports the hypothesis that testosterone mediates these sociosexual behaviors by activating cell nuclear androgen receptors. Likewise, if

estradiol mediates sociosexual behaviors in male rats through the activation of cell nuclear estrogen receptors, directly blocking estrogen receptors should also inhibit these behaviors. The steroidal antiestrogen, RU 58668 was chosen to test this hypothesis because it was effective in blocking estradiol's effects on both copulatory behavior and brain estrogen receptor binding in female rats. RU 58668 had no effect on the restoration of copulatory behavior or partner preference in testosterone-treated gonadectomized male rats, even though the level of brain cell nuclear estrogen receptor occupation was significantly reduced to the level found in gonadectomized males. However, the restoration of scent marking and 50 kHz vocalizations were impaired by RU 58668. Therefore, the activation of cell nuclear estrogen receptors is necessary for the restoration of some, but not all, sociosexual behaviors. Besides cell nuclear estrogen receptors, there are additional, but unknown, targets of estradiol that play a role in mediating copulatory behavior in adult male rats. This work indicates that the signals from multiple steroid signaling pathways converge in the regulation of sociosexual behaviors.

ACKNOWLEDGMENTS

This dissertation is dedicated to my family for providing me with the opportunity to attend graduate school and their steadfast support throughout my life, and to my wife Sharon for her love and patience.

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Chapter 1 - Project Overview

The overall goal of this project is to understand how gonadal steroid hormones influence the brains of adult male rats to modify the display of different sociosexual behaviors. The phrase sociosexual behavior is used here to refer to a number of steroid hormone-dependent social and reproductive behaviors (reviewed in Hart 1974), such as male copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking. The study of how gonadal steroids mediate these behaviors provides a starting point for understanding complex aspects of mammalian brain function. By analogy, gonadal steroids function as keys to unlock neural circuits that mediate hormone-dependent behavioral responses to an animal's environment (Pfaff 1989). Therefore, gonadal steroid hormones can be used to investigate the neural mechanisms responsible for these behaviors. The premise of this approach is to study the neural mechanisms responsible for these complex behaviors starting at the most fundamental level, the steroid hormones themselves. By identifying the specific hormones involved, where they are acting, and through what mechanisms, the neural substratum mediating hormone-dependent behaviors can be determined (Cottingham and Pfaff 1986).

Testosterone is believed to play a central role in mediating many sociosexual behaviors in adult male rats, especially male copulatory behavior. However, testosterone can be converted by enzymes in the brain to both dihydrotestosterone (an androgen) and estradiol (an estrogen) (see Figure 1). In male rats, dihydrotestosterone is not considered to have a major role in mediating male copulatory behavior, although this is not the case for estradiol. The role of estradiol in male rats was first established in the study of brain sexual differentiation, where the aromatization of testosterone to estradiol was found to be critical for masculinizing the brains of prenatal male rats. This served as the foundation for the aromatization hypothesis, which postulated that testosterone was just a precursor for the

formation of estradiol in the brain. Therefore, it was possible that some of the effects traditionally ascribed to testosterone could in fact have been mediated by estradiol.

For example, numerous studies have shown the importance of aromatizable androgens (i.e., ones that can be converted to estrogenic compounds, such as testosterone) in the regulation of male copulatory behavior. Nonaromatizable androgens (such as dihydrotestosterone (DHT), methyltrienolone (R1881), or fluoxymesterone) poorly restore copulatory behavior in adult male rats. In addition, treatment of gonadectomized male rats with estradiol alone has been reported to partially restore male copulatory behavior. Therefore, the aromatization of testosterone to estradiol is likely to play an important role in mediating male copulatory behavior in rats. However, the doses of exogenous estradiol needed to restore male copulatory behavior are well above the estradiol doses needed to achieve normal estrogen receptor occupation levels in intact male rats. This has led to the controversy concerning which steroid hormones (testosterone and/or estradiol) mediate

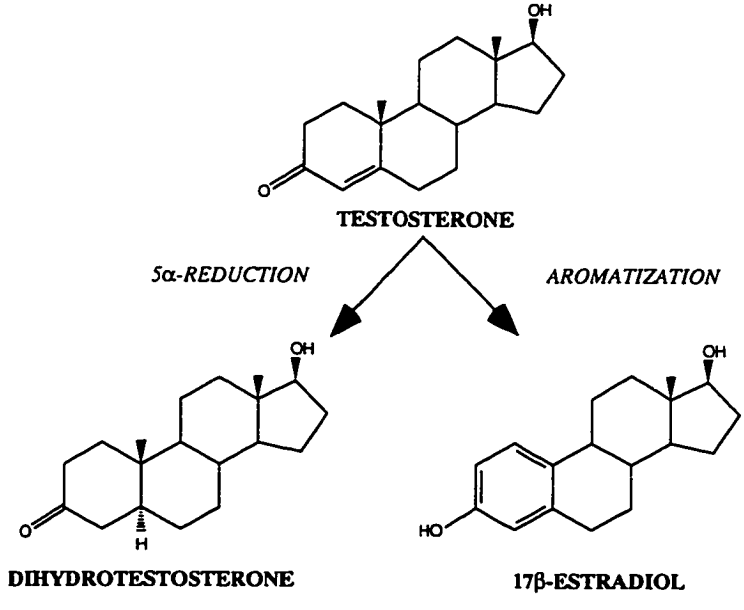


Figure 1. Aromatization and 5α-Reduction of Testosterone

male copulatory behavior in male rats.

The roles of testosterone and estradiol in mediating hormone-dependent sociosexual behaviors, such as male copulatory behavior and partner preference, have served as the basis for this project. To that end, the following four specific aims were first proposed:

1. To assess the relative contribution of androgen receptor action, aromatization, and estrogen receptor action in the regulation of male rat sexual behavior.
2. To compare the hormonal specificity of aromatase and androgen receptor mRNA regulation in selected regions of the adult male rat brain.
3. To determine if hormone treatment alters mRNA stability or transcriptional regulation of the androgen receptor and aromatase messages in rat brain tissue in vivo.
4. To determine if hormone treatment alters the protein stability of rat brain androgen receptors in vivo.

However, research does not often follow a linear path, and this work is a good example. After beginning the project, it soon became clear that specific aim #1 was going to be a much greater undertaking than was initially anticipated, and my advisory committee felt that specific aim #1 should take precedence over the others. This decision was also influenced by finding that other laboratories had published major parts of specific aim #2 before I was able to (these studies are discussed in the next chapter in the sections concerning the regulation of aromatase and androgen receptors). Therefore, I reorganized my specific aims, with the assistance and approval of my doctoral committee, by expanding the first aim into a project of its own.

This project is centered around the following questions: Is estradiol (produced from the aromatization of testosterone) necessary for the restoration of male copulatory behavior and partner preference? Are different hormone-dependent sociosexual behaviors (including male copulatory behavior, partner preference, scent marking, and 50 kHz ultrasonic vocalizations) differentially regulated by testosterone and/or estradiol? What are the relative

contributions of testosterone and estradiol in mediating sociosexual behaviors? Are cell nuclear androgen receptor and estrogen receptor action necessary for the restoration of different sociosexual behaviors? Does cell nuclear androgen receptor action contribute to the role of cell nuclear estrogen receptor action in the restoration of sociosexual behaviors?

Specific Aim #1: To block testosterone's restoration of male copulatory behavior and partner preference by inhibiting brain aromatase.

Specific Aim #2: To block testosterone's restoration of hormone-dependent sociosexual behaviors by directly inhibiting estrogen receptors with chronic antiestrogen treatment.

Specific Aim #3: To block testosterone's restoration of hormone-dependent sociosexual behaviors by directly inhibiting androgen receptors with chronic antiandrogen treatment.

Specific Aim #4: To assess the relative contribution of androgen receptor and estrogen receptor action on the regulation of hormone-dependent sociosexual behaviors in male rats by the combined systemic administration of an antiandrogen and an antiestrogen.

Chapter 2 - Historical Introduction

History of Gonadal Steroid Hormones

The effects of gonadal hormones on physiology and behavior have been recognized since ancient times, with the practice of removing the gonads of animals for domestic use documented in Asia Minor over 6000 years ago (reviewed in Mooradian et al. 1987, Kochakian 1990). This historical evidence indicates that the links between the testes and fertility, secondary sex characteristics, and behavior, have been known for a long time. However, proof that the testes produce a substance, which travels throughout the bloodstream, only came relatively recently (reviewed in Beach 1981, Kochakian 1993).

In one of the earliest known endocrinology experiments, Berthold (1849) showed that the behavioral (such as crowing and sexual interest in hens) and physical (such as comb and wattle size) characteristics of gonadally intact male roosters could be maintained after gonadectomy, by reimplanting the rooster's own testes into another body cavity (reviewed in Forbes 1949). Without reimplanting the testes, the roosters gradually stopped crowing and did not mate. This experiment indicated that a blood-borne substance, produced by testes, was necessary to maintain the behavioral and physical characteristics of intact male roosters (reviewed in Kochakian 1990). This experiment was important because Berthold observed the animals' behavior, as well as their physical characteristics, throughout his experiments (reviewed in Beach 1975). Further support for the hypothesis that the testes were a secretory organ came from Brown-Sequard who injected aqueous extracts of animal testes into himself and reported a number of benefits ranging from increased muscle strength to improved mental faculties (Brown-Sequard 1889).

During the latter half of the nineteenth century, the links between hormonal secretions and the regulation of behavior by the nervous system were established (reviewed

in Beach 1981). However, there was debate as to whether the effects of hormones on the brain and behavior were mediated directly by the action of hormones on the brain, or indirectly by the action of hormones on peripheral tissues, such as the seminal vesicles. This debate was largely settled by the work of Steinach (1894), who was a strong proponent of a "centralistic theory of hormone action" in the nervous system. Steinach showed that the removal of the sex accessory organs of male frogs, such as the seminal vesicles, did not have a negative effect on copulatory behavior (reviewed in Steinach and Leobel 1940). Therefore, the seminal vesicles were not responsible for transmitting the hormonal signal, and the connection between the brain and testicular secretions was established for its role in mediating behavior.

After it was understood that the changes in the behavior and physical characteristics of an animal after gonadectomy were due to the loss of a blood-borne substance produced in the testes, work progressed towards the substance's identification. Analysis of testicular extracts and male urine led to the discovery of androgens in the 1930s (reviewed in Kochakian 1993). The first androgen isolated in pure crystalline form was androsterone (Butenandt 1931). This initiated the first detailed chemical analysis of male sex hormones and showed that androsterone contained a cholesterol-like ring structure. Shortly thereafter, testosterone was isolated, and then synthesized from cholesterol (David et al. 1935). After it was realized that several compounds possessed the characteristics of a male sex hormone, besides testosterone, the generic name "androgen" (andro = male, gen = to produce) was given to the family of C_{19} steroids that contained cholesterol-like ring structures.

Testosterone, produced in the testes, plays an important role in mediating reproductive physiology and behavior (Rezek and Whalen 1978, Green and Leake 1987, Mooradian et al. 1987). Shortly following gonadectomy, serum testosterone levels are greatly reduced (by at least 20-fold), and remain low even 1 year later (Coyotupa et al. 1973, Ando et al. 1986). The adrenal glands also produce androgens, but these are not sufficient for the maintenance of male copulatory behavior or prostate/seminal vesicle size

in gonadectomized adult rats (Poggioli et al. 1984). Once secreted into the bloodstream, gonadal steroid hormones bind to a carrier protein called sex hormone binding globulin (Hammond and Bocchinfuso 1996). In the blood, free gonadal steroids circulate in equilibrium with sex hormone binding globulin-bound gonadal steroids before they diffuse into cells.

Brain Androgen Metabolism

The metabolism of testosterone, within or near its target-tissues, represents a way for those tissues to directly control the actions of this signaling molecule (Stewart and Scheppard 1992). Testosterone can be enzymatically converted into at least 27 compounds in most species (reviewed in Kochakian 1993). However, not all of these compounds are believed to play substantial roles, and most are thought to be part of inactivation pathways (Vida 1969, Heftmann 1970). Many of the enzyme reactions that metabolize testosterone are reversible, such as in the dehydrogenase reactions (see Appendix A, Figure 50). This has made the study of testosterone's functions more complex because many of its metabolites are interconverted in vivo. In addition, there are subtle, yet significant, sex and species differences in brain testosterone metabolism (reviewed in Martini et al. 1990). For instance, 5α-reduction is a major route of testosterone metabolism in mammalian brains, while 5β-reduction is more common in many avian species (Massa et al. 1979, Balthazart et al. 1983).

In the adult male rat brain, dihydrotestosterone produced by 5α-reduction, and 17β-estradiol produced by aromatization are the two most abundant testosterone metabolites (see Figure 1). For instance, shortly after the injection of radiolabeled testosterone into gonadectomized/adrenalectomized adult male rats, 98% of the radioactivity can be extracted from limbic brain cell nuclei as dihydrotestosterone (20.8%), estradiol (53.5%), and unmetabolized testosterone (23.7%) (Lieberburg and McEwen 1977). Therefore, many studies in rodents have focused on the roles of 5α-reduction (reviewed in Martini 1982) and aromatization (reviewed in Lephart 1996).

5α-Reduction

The membrane-bound enzyme, 5α-reductase, irreversibly catalyzes the NADPH-dependent reduction of steroids with 3-keto, Δ4,5 double bonds (Martini 1982, Russell et al. 1994). Testosterone and androstenedione are two major substrates for 5α-reductase, which result in the formation of dihydrotestosterone and androstandione, respectively. In adult male rat serum, the approximate ratio of testosterone to dihydrotestosterone is 25:1 (Saksena and Lau 1979). Both testosterone and dihydrotestosterone bind and activate cell nuclear androgen receptors (this topic is covered later on page 28). Initially, it was thought that 5α-reduction was an inactivation pathway for testosterone (Heftmann 1970). However, 5α-reduction is now considered an amplification mechanism, by preventing the formation of inactive testosterone metabolites (George 1997). One of the most accepted functions for 5α-reductase activity is in maintaining tissues of the male reproductive tract and male secondary sex characteristics, where dihydrotestosterone has been shown to be the primary effector in rats (Baulieu et al. 1968).

5α-reductase activity has been documented in peripheral tissues such as testes, prostate, seminal vesicles, adrenal glands, kidneys, liver, and sebaceous glands (Martini et al. 1990), along with central nervous structures such as the hypothalamus, midbrain, amygdala, hippocampus, cerebellum, and cerebral cortex (Krieger et al. 1983, Celotti et al. 1992). The expression of brain 5α-reductase activity does not appear to be sexually dimorphic (Melcangi et al. 1987), or under androgenic control because gonadectomy does not change 5α-reductase activity (Melcangi et al. 1985). There are two different genes for 5α-reductase, designated type #1 and type #2, which have been cloned in rats and humans (Russell et al. 1994). The proteins these genes encode have different substrate affinities (the rat type #1 5α-reductase has approximately a 10-15 fold lower affinity for testosterone than the rat type #2 5α-reductase does) and different pH optima (the rat type #1 5α-

reductase has a neutral to basic pH optimum and the rat type #2 5α-reductase has an acidic pH optimum) (Normington and Russell 1992).

Analysis of mRNA and 5α-reductase activity has shown that type #1 5α-reductase is the predominant form in the rat brain (Normington and Russell 1992), and 5α-reductase type #2 mRNA is not expressed in the mouse brain (Mahendroo et al. 1996). However, detailed neuroanatomical studies using either in situ hybridization or immunocytochemistry have not been conducted, so there is the possibility that some type #2 5α-reductase could be expressed in the brain. In comparing different roles of both 5α-reductase isozymes, type #1 is thought to have a primarily catabolic role, while type #2 is thought to have a primarily anabolic role in maintaining male secondary sex characteristics (Normington and Russell 1992). For a more in depth review of 5α-reductase and its role in neuroendocrine function, see Martini (1992).

It should also be noted that 5α-reductase activity is often associated with 3α(β)-hydroxysteroid dehydrogenase activity that reversibly converts dihydrotestosterone into 5α-androstan-3α-17β-diol (3α-diol) and 5α-androstan-3β-17β-diol (3β-diol) (Melcangi et al. 1993). Interestingly, estrogen receptors have a moderate affinity for 3β-diol, but not its stereoisomer, 3α-diol (Vreeburg et al. 1975). Therefore, the metabolism of dihydrotestosterone to 3β-diol, which activates estrogen receptors, might be responsible for the estrogenic effects on copulatory behavior observed in gonadectomized male rats treated with dihydrotestosterone (Morali et al. 1994).

Aromatization

The biosynthesis of estrogen has been well conserved throughout evolution and occurs in all vertebrates (Callard et al. 1990, Simpson et al. 1994). The process of aromatization of testosterone to 17β-estradiol is accomplished by a complex of three microsomal enzymes; cytochrome P450 aromatase, P450 XIXA1, and cytochrome P450

reductase. The central protein in this reaction, cytochrome P450 aromatase (hereafter denoted as aromatase), is involved in both substrate binding and catalysis (Lephart 1996). The other two enzymes in the complex (P450 XIXA1 and cytochrome P450 reductase) are ubiquitously expressed in most cells and are not believed to have any regulatory roles on aromatase activity. This enzyme complex irreversibly converts C_{19} androgens with unsaturated steroid A-rings into C_{18} estrogens with saturated steroid A-rings. So, 17β-estradiol cannot be converted back to testosterone. Dihydrotestosterone cannot be aromatized because it already has a saturated steroidal A-ring. Therefore, these two major routes for testosterone metabolism (5α-reduction and aromatization) are one-way processes, resulting in the formation of either dihydrotestosterone or 17β-estradiol (see Figure 1). For a detailed description of aromatase biochemistry, gene structure and expression, see Chen et al. (1993), Simpson et al. (1994), and Lephart (1996).

Localization of Cytochrome P450 Aromatase

The most common sites of aromatase activity in rats, measured in biochemical assays that monitor the conversion of androgens to estrogens, are the gonads and the brain (reviewed in Simpson et al. 1994, Lephart 1996). However, in humans aromatase has also been identified in skin fibroblasts, placenta, and adipose tissue (Harada et al. 1990). In rats, aromatase is restricted to the brain and the gonads (Lephart et al. 1990, Fitzpatrick and Richards 1993, Yuan et al. 1995). Even though the testes can produce estradiol, gonadectomy does not change serum estradiol levels in rats (Abdelgadir et al. 1994). In addition, serum testosterone levels decrease with age in male rats, but serum estradiol levels do not (Winters and Takahashi 1983).

The first direct biochemical evidence for aromatization in the central nervous system came from studies on human fetal diencephalon tissue (Naftolin et al. 1971). Shortly thereafter, this work was also carried out in rats, and aromatase activity was shown to

occur in the anterior hypothalamus (Naftolin et al. 1972). When systemic injections of tritiated-testosterone are given to male rats, tritiated-estradiol can be extracted from limbic brain tissue (pooled hypothalamus, preoptic area, amygdala, and septum), but not from the pituitary (Lieberburg and McEwen 1975). A similar study, which examined individual adult male rat brain nuclei, showed that tritiated-estradiol (converted from tritiated-testosterone) could be isolated from the amygdala, preoptic area, hypothalamus, and septum, but not from the pituitary (Lieberburg and McEwen 1977). Other work also has shown that the pituitary does not contain aromatase activity (Naftolin et al. 1975, Roselli et al. 1984).

The neural localization of aromatase activity has been phylogenetically conserved (Callard et al. 1978). All the animals listed in Table 1 share similar patterns of aromatase expression with rats. In adult male rats, brain regions that express aromatase are listed in Table 2. The highest levels of aromatase mRNA (determined by RNase protection assays, Abdelgadir et al. 1994) and enzyme activity (Roselli et al. 1984) in the adult male rat brain are generally found in the amygdala and the preoptic area-hypothalamus. Within the preoptic area, aromatase mRNA is primarily expressed in the sexually dimorphic nucleus and not in the medial preoptic nucleus, the periventricular preoptic nucleus, or the subfornical organ (Lauber and Lichtensteiger 1994). Immunolocalization studies (Sanghera et al. 1991, Jakab et al. 1993, Shinoda et al. 1994) have generally produced similar results to those described above, but the most credible evidence for the neural localization of aromatase still has come from directly measuring aromatase enzyme activity with biochemical assays (Lephart 1996). In conclusion, it is believed that the most important sites of aromatase activity in adult male rats are located in the amygdala and the preoptic area-hypothalamus.

Table 1. Animals with Similar Patterns of Brain Aromatase Expression

Animal	Reference
Atlantic salmon	(Andersson et al. 1988)
chicken	(Beyer et al. 1994)
cynomolgus monkey	(Resko et al. 1993)
dove	(Hutchison et al. 1986)
ferret	(Carroll et al. 1988)
goldfish	(Pasmanik et al. 1988)
guinea pig	(Connolly et al. 1990)
hamster	(Hutchison et al. 1991)
human	(Honda et al. 1994)
Japanese monkey	(Yamada-Mouri et al. 1995)
Japanese quail	(Balthazart et al. 1991)
mouse	(Yamada et al. 1993)
musk shrew	(Dellovade et al. 1994)
opossum	(Fadem et al. 1993)
rabbit	(George et al. 1978)
rat	(Lephart 1996)
zebra finch	(Shen et al. 1995)

Table 2. Neural Localization of Male Rat Brain Aromatase

Region	Relative Abundance	Reference
amygdala	+++	a, b, c
bed nucleus of the stria terminalis	+++	c
cerebellum	ND	a
cingulate cortex	ND	a
hippocampus	+	a, b
lateral septum	+	d
medial basal hypothalamus	++	a, b, c
parietal cortex	ND	a
preoptic area	++	a, b, c

ND	Not Detected	
a	mRNA level measured by RNase protection assay	(Abdelgadir et al. 1994)
b	aromatase enzyme activity	(Roselli et al. 1984)
c	mRNA level measured by in situ hybridization	(Lauber and Lichtensteiger 1994)
d	aromatase immunolocalization	(Jakab et al. 1994, Shinoda et al. 1994)

Regulation of Cytochrome P450 Aromatase

The regulation of cytochrome P450 aromatase represents an important step in controlling the neural effects of androgens. Control of the intracellular ligand supply by metabolic enzymes provides the cell with a way to modulate a hormonal signal directly (Stewart and Scheppard 1992). Adult male rats express more aromatase activity in the preoptic area-hypothalamus than females (Roselli et al. 1984, Steimer and Hutchison 1990, Von Ziegler and Lichtensteiger 1992). This sexually dimorphic expression of aromatase activity has been attributed to sex-differences in circulating androgen levels. There are also sex-differences in the ability of androgens to induce the expression of aromatase activity in different brain regions, which could contribute to the ability of androgens to stimulate male copulatory behavior in females (Roselli et al. 1996).

Testosterone increases both aromatase mRNA levels and enzyme activity in the adult male rat medial basal hypothalamus and preoptic area, but not in the amygdala (Abdelgadir et al. 1994). Within the amygdala, there are two discrete populations of aromatase containing neurons; a gonadectomy-sensitive population in the medial amygdala, and a gonadectomy-insensitive population in the cortical amygdala (Wagner and Morrell 1996). This regional difference in the hormonal specificity of aromatase regulation is very interesting because it also corresponds to region-specific differences in the developmental pattern of aromatase expression (Shinoda 1994, Shinoda et al. 1994). Therefore, the adult male rat brain could possess two distinct aromatization systems that could each play unique roles in the regulation of male reproductive behavior: a gonadectomy-sensitive "hypothalamic" system and a gonadectomy-insensitive "limbic ring" system, shown in Figure 2 (Jakab et al. 1993).

Aromatase mRNA levels are up-regulated by androgen treatment in wild type adult male rats. Preoptic area aromatase mRNA levels in male rats with genetic androgen receptor

mutations (tfm/Y) are at the same levels as wild type gonadectomized male rats, and testosterone administration has no effect on tfm/Y androgen receptor mRNA levels (Olsen 1992). This indicates that androgen receptors have a role in the regulation of adult rat brain aromatase mRNA. Testosterone regulates aromatase activity by altering its steady-state mRNA levels presumably by transcriptional activation and/or altering mRNA stability (Berkovitz et al. 1992, Abdelgadir et al. 1994). In addition, there is also evidence for posttranscriptional regulation of aromatase, because estradiol increases aromatase enzyme activity, but does not alter aromatase mRNA levels in the preoptic area of adult male rats (Abdelgadir et al. 1994). However, the precise mechanisms for the androgen-dependent regulation of aromatase mRNA levels are not known at this time.

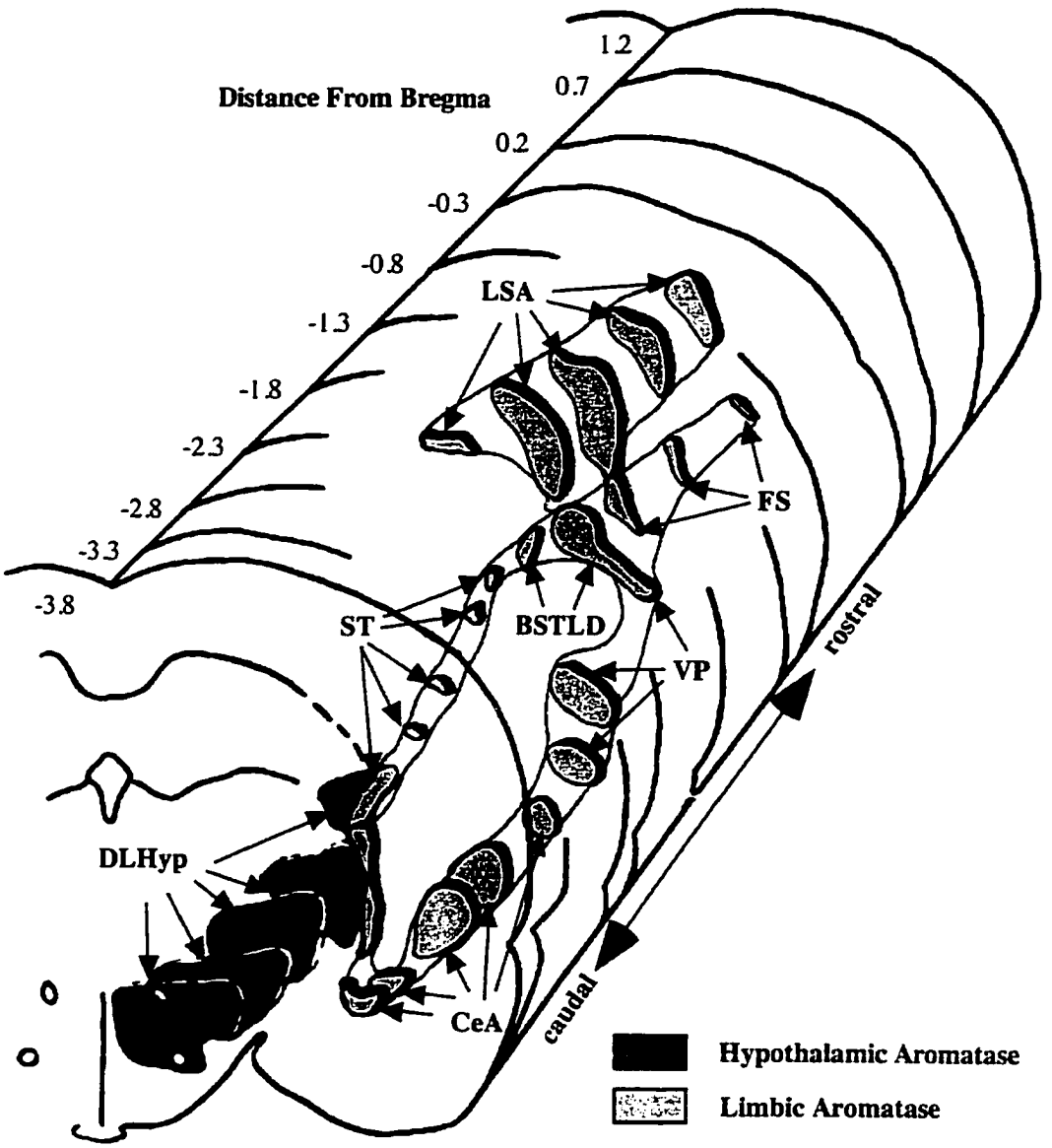


Figure 2. Representation of Brain Aromatase Immunolocalization

Reprinted from the Journal of Steroid Biochemistry and Molecular Biology, Volume 44, Jakab, R. L.; Horvath, T. L.; Leranth, C.; Harada, N.; and Naftolin, F. Aromatase immunoreactivity in the rat brain: Castration-sensitive hypothalamic neurons and an unresponsive "limbic ring" of the lateral septum-bed nucleus-amygdala complex. Pages 481-98. Copyright 1993, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK. The areas in black denote sites of gonadectomy-sensitive aromatase immunoreactivity in the adult male rat hypothalamus, labeled as dorsolateral hypothalamus (DLHyp). The areas in grey denote sites of gonadectomy-insensitive aromatase immunoreactivity in the limbic system. The "limbic ring" structures labeled are the central amygdala (CeA), ventral pallidum (VP), stria terminalis (ST), fundus striati (FS), lateral-dorsal portion of the bed nucleus of the stria terminalis (BSTLD), and the lateral septal area (LSA).

Mechanisms for Gonadal Steroid Hormone Action

The foundation for our understanding of steroid hormone action is that steroid hormones influence cells by altering gene expression, which was first proposed by Karlson (1961, 1963) and Zalokar (1961). This hypothesis, called the "hormone-gene thesis" (Hechter and Halkerston 1965), is now widely accepted (Yamamoto 1985). In a review of this subject over 30 years ago, Hechter and Halkerston (1965 p. 134) presented a question that is still pertinent today: ". . . where steroid hormone effects are observed, or inferred, at the gene locus, does steroid act directly or indirectly with a component of the regulatory system?" Changes in gene expression mediated by intracellular receptors are thought to be responsible for the behavioral effects of steroid hormones (McEwen 1988, McEwen et al. 1977). However, it is not known whether all the effects of steroid hormones on behavior are mediated exclusively by intracellular receptors regulating gene transcription (Pfaff 1996). Therefore, the fundamental mechanisms of steroid hormone action are germane to the study of male hormone-dependent sociosexual behaviors. In addition, this topic touches upon a central theme of this dissertation, and is embodied in the question: Are the effects of gonadal steroid hormones on hormone-dependent sociosexual behaviors mediated by the actions of cell nuclear steroid receptors, or by other mechanisms?

The role of intracellular receptors in mediating the effects of steroid hormones has been supported by three lines of evidence (McEwen et al. 1977):

1. There is a long time-lag involved in generating a response after the hormone is administered.
2. RNA and protein synthesis inhibitors block the effects of steroid hormones.
3. Steroid hormone antagonists inhibit the actions of hormones by blocking their receptors.

However, there is a growing appreciation for diverse mechanisms of steroid hormone action (Brann et al. 1995). Therefore, it is important to consider whether the effects of

steroid hormones are mediated by classical mechanisms of steroid hormone action (discussed in the following section), or by nonclassical mechanisms of steroid hormone action (discussed on page 34), which can also result in the alteration of gene expression. The nomenclature of classical and nonclassical steroid hormone action has been adapted from Mahesh et al. (1996) and Orchinik and McEwen (1993). The phrases "intracellular receptors" and "nuclear steroid receptors" refer to proteins in the steroid hormone receptor superfamily of ligand-inducible transcription factors, and should not be confused with other steroid hormone binding proteins.

Classical Mechanisms - Nuclear Steroid Receptor Action

Steroid hormones bind to a family of selective intracellular receptors that act as ligand-inducible transcription factors (O'Malley and Tsai 1992, Power et al. 1992, McDonnell et al. 1993). Figure 3 depicts a simplified model for steroid hormone receptor action (Landers and Spelsberg 1991, Tsai and O'Malley 1994). Steroid hormones enter cells by diffusion through the plasma membrane (Landers and Spelsberg 1991), but facilitated transmembrane steroid transport has also been postulated to play a role in mediating intracellular steroid levels (Thompson 1995). Once inside the cell, steroids then bind to high-affinity receptors that are found in complexes with other proteins (Smith and Toft 1993). Nuclear steroid receptors (in both the unbound and ligand-bound states) are associated with a number of heat-shock (HSP 56, 70, & 90) and other proteins. These steroid receptor-associated proteins are believed to have important roles in ligand binding, protein folding, and intracellular localization. For further discussion on these steroid receptor-associated proteins, see the following reviews: Hutchison et al. (1993), Caamano et al. (1994), and Pratt and Welsch (1994).

The process of steroid binding, leading to active receptor complexes, has been called "receptor transformation" (Jensen et al. 1974, Baulieu 1975). This phrase originated

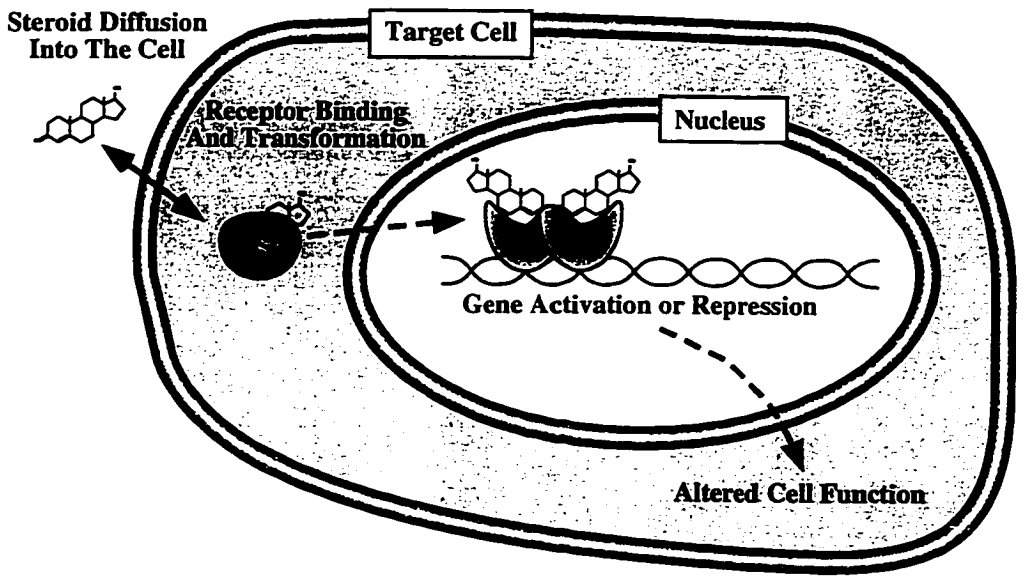


Figure 3. Basic Model For Steroid Hormone Receptor Action

from radioligand-binding studies, where it was found that the mobility of steroid hormone receptor/radioligand complexes, purified in sucrose density gradients, differed depending on whether the samples were prepared from cytosol or nuclear fractions (Toft and Gorski 1966, Stancel and Gorski 1975, Traish et al. 1985). For example, dihydrotestosterone binding resulted in the transformation of a cytoplasmic 7-8 S sedimenting androgen receptor-protein complex to a nuclear 4-5 S sedimenting androgen receptor-protein complex (Traish et al. 1985). This is explained by the fact that steroid hormone binding results in a conformational change in the receptor, which exposes a dimerization domain within the "hinge region" of the receptor and also a change in proteins associated with the receptor, such as the ligand-dependent release of HSP 90 (Landers and Spelsberg 1991).

Steroid receptor complexes, without bound ligands, can be located in either the cytosol or nucleus, depending on the type of steroid hormone receptor in question

(Welshons et al. 1984, Wilstrom et al. 1987). In the case of cytosolic steroid receptors (notably androgen and glucocorticoid receptors), hormone binding facilitates the exposure of a nuclear localization signal for ligand-dependent import into the nucleus (Picard et al. 1990, Pratt et al. 1993, Defranco et al. 1995). Biochemical assays have shown that androgen receptors can be detected in cytosolic fractions in the absence of hormone, and then following hormone addition, the receptors are found mainly in the nucleus (McGinnis et al. 1983, Hoppen and Hammann 1987, Kemppainen et al. 1992). On the other hand, estrogen and progestin receptors are predominantly nuclear proteins that do not undergo ligand-dependent changes in subcellular localization (Gorski et al. 1984, Welshons et al. 1984, Ylikomi et al. 1992).

The nuclear steroid receptors all have highly conserved zinc-finger DNA binding domains, and they have been grouped into the nuclear receptor superfamily (Freedman 1992, Parker 1993b). Within this superfamily, the two proteins to be discussed in further detail are androgen receptors and estrogen receptors. The general domain structure of nuclear steroid receptors is depicted in Figure 4. The homologies between different members of the nuclear receptor superfamily have been covered in the following reviews, which provides a perspective on their evolutionary origin: Yamamoto (1985), Amero et al. (1992), Baker (1992), Ojasoo et al. (1994), and Sumida (1995).

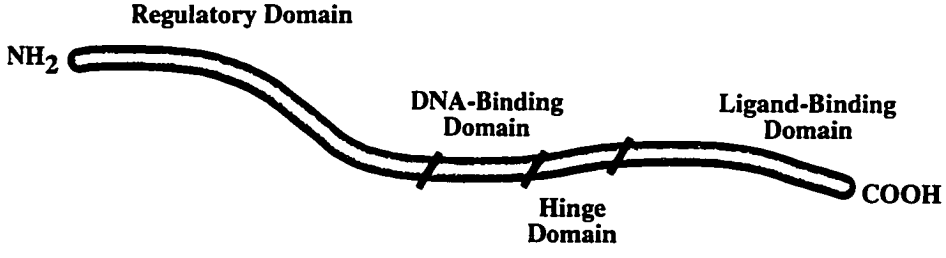


Figure 4. Functional Domains of Nuclear Steroid Receptors

Steroid hormone receptors regulate (either positively or negatively) the transcription of target genes. Dimers of ligand-activated steroid hormone receptors (containing zinc-finger DNA binding domains) specifically bind regulatory sequences on genomic DNA, called hormone response elements (Loreni et al. 1988, Landers and Spelsberg 1991, Van Dijck and Verhoeven 1992, Warriar et al. 1993). Hormone response elements are palindromic sequences (see Figure 5) located most often upstream of the transcription start site, but they also can be found within a gene's introns or exons (Claessens et al. 1990). The binding of steroid hormone receptors to hormone response elements, in conjunction with other protein-protein interactions (Truss and Beato 1992, Smith et al. 1997), results in a conformational change in the DNA that can have either positive or negative effects on transcription (Parker 1990, Metsis et al. 1992).

Dimers of androgen receptors, glucocorticoid receptors, and progestin receptors all bind the same palindromic consensus sequence (the hormone response element), yet each only activate specific genes in select tissues (Truss and Beato 1992). Dimers of estrogen receptors bind to a similar consensus sequence termed the estrogen response element (see Figure 5), which differs from the hormone response element in only 5 bases (Zilliacus et al. 1995). The selective induction of genes containing hormone response elements, by different steroid receptors, is likely to be due to several factors including differences in the steroid receptors' DNA-binding domains, and interactions with nonreceptor factors (De-Vos et al. 1994, Robins et al. 1994, Rundlett and Miesfeld 1995). Moreover, subtle conformational changes in steroid receptors induced by different ligands have been shown

	Consensus Sequence
Hormone Response Element	G G T A C A n n n T G T T C T
Estrogen Response Element	A G G T C A n n n T G A C C T

Figure 5. Hormone/Estrogen Response Elements

to have distinct effects on DNA binding and activation (Allan et al. 1992, Vegeto et al. 1992, Veldscholte et al. 1992).

It should be noted that steroid receptors can also be activated in a ligand-independent manner (i.e., without steroid hormones) (O'Malley et al. 1995). For instance, epidermal growth factor (EGF) has been shown to activate estrogen receptor-dependent transcription of reporter gene constructs containing estrogen response elements (EREs), without estradiol in cultured cells (Ignar-Trowbridge et al. 1993). Furthermore, cellular signal transduction pathways (such as cAMP signaling) can also modulate the action of nuclear receptors (Nordeen et al. 1994, Katzenellenbogen 1996, El-Tanani and Green 1997), which has been shown to be behaviorally relevant. For instance, Mani et al. (1994) have shown that the administration of D_1, but not D_2, dopamine receptor agonists stimulates progestin receptor-dependent behaviors in female rats. One important aspect of this experiment was that this effect was mediated by the activation of progestin receptors in the absence of progesterone. Therefore, steroid receptor signaling can occur even in the absence of a hormonal stimulus. However, it is not known how common a mechanism this is, relative to the traditional steroid hormone-dependent activation of nuclear receptors.

Estrogen Receptors

Receptors that bind radiolabeled estrogens (estrophilins) were first detected in female reproductive tissues (Toft and Gorski 1966). Later studies showed that radiolabeled estrogens also accumulate in male reproductive tissues (Robinette et al. 1978). Both female and male brains express specific high affinity estrogen receptors (Kato and Villee 1967, Vreeburg et al. 1975). The development of radioligand binding assays for estrogen receptors, isolated in sucrose density gradients (Toft and Gorski 1966, Stancel and Gorski 1975) or localized by dry-mount autoradiography (Stumpf and Roth 1966, Zigmond and McEwen 1970), were two major technical advances in the localization of steroid receptors.

These two techniques were complimentary to each other, and showed that steroid hormone binding resulted in the formation of tightly associated nuclear receptor complexes (Sar and Stumpf 1973, Stumpf and Sar 1976). Competitive binding studies and Scatchard analyses have revealed that estrogen receptors exhibit a high affinity for estrogens (~0.2 nanomolar) with similar pharmacological characteristics in different tissues (Raynaud and Moguilewsky 1977, Lieberburg et al. 1980, Muldoon et al. 1988, Ojasoo et al. 1994, Kuiper et al. 1997). These receptors are highly selective for estrogens, with a very low affinity for testosterone, progesterone, or corticosterone (Mercier et al. 1976, Saiduddin and Zassenhaus 1977). They are also stereospecific, having a much higher affinity for 17β-estradiol than for 17α-estradiol (Clark et al. 1982b).

Not long ago, it was thought that only single genes encoded each gonadal steroid hormone receptor. For instance, only single genes had been identified for androgen and progestin receptors. However, for other members of the nuclear receptor superfamily, such as the retinoic acid receptors, multiple subtypes had been identified (Amero et al. 1992). Recently, a novel form of the rat estrogen receptor, designated estrogen receptor-beta, was cloned (Kuiper et al. 1996). This new member of the nuclear hormone receptor superfamily was found to have considerable sequence homology to the originally identified estrogen receptor, now designated estrogen receptor-alpha. These proteins shared greater than 95% amino acid identity in the DNA-binding domain, but diverged in the C-terminal ligand-binding and the N-terminal regulatory domains. More importantly, this protein was shown to activate reporter gene constructs containing consensus estrogen receptor elements in a ligand-dependent manner (Kuiper et al. 1996).

Very little is known about this newly identified estrogen receptor, except that it has been postulated that it could be responsible for the residual estradiol binding capacity in mice with disruptions in the estrogen receptor-alpha gene (Katzenellenbogen 1997). The activity of this second estrogen receptor could also explain why deleting estrogen receptor-alpha is not lethal (Korach et al. 1996). The following discussion refers primarily to the

estrogen receptor-alpha, because so little is known at this time about estrogen receptor-beta. The receptor subtype designation will not be used, except when it is known which receptor is responsible, because it is likely that many of the effects previously thought to be mediated by estrogen receptor-alpha are actually mediated by estrogen receptor-beta.

The molecular cloning of the gene encoding estrogen receptor-alpha from different species revealed that this protein has been highly conserved across different phyla (Greene et al. 1986, Koike et al. 1987, Weiler et al. 1987, White et al. 1987, Todo et al. 1996). The rat estrogen receptor-alpha gene encodes a single 67 kilodalton polypeptide that shuttles between the cytosol and the nucleus, with the equilibrium favoring nuclear localization (Koike et al. 1987, Picard et al. 1990). Just like cytochrome P450 aromatase, transcription from the estrogen receptor gene occurs from multiple promoters that may be involved in its tissue-specific expression. Recently, it has been shown that there are at least three different 5' untranslated regions of estrogen receptor-alpha exon #1 in rats, and that "exon ON" is only expressed in peripheral tissues (Hirata et al. 1996a, Hirata et al. 1996b). In addition, complex patterns of estrogen receptor mRNA splicing have been observed in humans that result in a number of different estrogen receptor isoforms (Sluyser 1994, Leygue et al. 1996). Estrogen receptor variants have also been detected in the rat brain and pituitary that are missing exon #4, which encodes part of the hormone binding domain (Skipper et al. 1993, Friend et al. 1995). Deletion of exon #4 results in a constitutively active estrogen receptor. The expression of this estrogen receptor variant was also found to be estrogen-dependent in the pituitary. Therefore, mRNA splicing could play a significant role in mediating estrogen receptor action, but the physiological relevance of these variants to brain function is not known.

The estrogen receptor has a characteristic steroid receptor domain structure (see Figure 4). Unlike other members of the steroid hormone superfamily, the estrogen receptor is believed to have two transcriptional activation domains, TAF-1 and TAF-2. TAF-1 is located in the N-terminal portion of the protein and is ligand-independent, and TAF-2 is

located in the within the C-terminal hormone-binding domain and is ligand-dependent (Parker 1993c). It is possible that other receptors share this structure, but this topic has not been extensively researched. The interplay between these two transcriptional activation domains with other trans-regulatory factors and cis-acting DNA sequences on estrogen-responsive genes is believed to determine whether target gene transcription is activated or repressed (Parker 1993c, Katzenellenbogen 1996). Knowledge of these domains has helped in the understanding of why some estrogen receptor antagonists (antiestrogens) are partial agonists (McDonnell et al. 1995, Jensen 1996, Katzenellenbogen 1996).

The progestin receptor is a good example of an estrogen-dependent gene. The neural induction of the progestin receptor by estrogens is critical for the expression of female copulatory behavior (Edwards et al. 1968, Whalen 1974, Tennent et al. 1980, McGinnis et al. 1981, Pleim and Barfield 1988). Both male and female rats express estrogen and progestin receptors in similar brain regions. However, sex differences have been reported in the ability of estradiol to upregulate the levels of cytosolic progestin binding (Etgen 1985, Brown et al. 1987) and steady-state progestin receptor mRNA (Lauber et al. 1991b) in the rat brain. On the other hand, estrogens equally regulate other genes in the liver of both male and female rats (Dickson and Eisenfeld 1979). These data indicate that the regulation of estrogen-responsive genes can occur in a sex and tissue-specific manner.

Estrogen Receptor Localization

Estrogen receptors are expressed in the brains of both female and male rats (Kranzler et al. 1984, Simerly et al. 1990, Yuan et al. 1995). Estrogen receptors are also expressed in female reproductive tissues (ovary (Saiduddin and Zassenhaus 1977) and uterus (Sar and Parikh 1986)) and male reproductive tissues (testes (Robinette et al. 1978) and seminal vesicles (West et al. 1990)); as well as other tissues including liver (Francavilla

et al. 1984), kidney (Winters and Takahashi 1983), adipose tissue (Gray et al. 1981), pituitary (Pelletier et al. 1988), and blood vessels (Knauthe et al. 1996). However, the most detailed neural estrogen receptor localization studies (measuring ligand-binding (Pfaff and Keiner 1972, Rainbow et al. 1982b), immunoreactivity (Blaustein 1992, Kallo et al. 1992), or mRNA levels (Simerly et al. 1990)) have been carried out in female rats only. Overall, the highest levels of neural estrogen receptors (measured by nuclear exchange assays on micropunched brain tissue from male or female rats) are found (in descending order) in the periventricular preoptic area, arcuate nucleus-median eminence, medial preoptic area, periventricular anterior hypothalamus, ventromedial hypothalamus, and medial amygdala (Kranzler et al. 1984).

When male rats are compared with female rats, estrogen receptors are found generally in the same brain regions (Simerly et al. 1990, Gibbs 1996, Diano et al. 1997). However, there are appreciable sex differences in the magnitude of brain estrogen receptor expression (Kranzler et al. 1984). Sex-differences in the levels of estrogen receptor expression are highly dependent upon gonadal steroid hormones during the early postnatal period; rather than innate genetic differences (Don Carlos et al. 1995). For example, males gonadectomized at birth express higher levels of estrogen receptors in the ventromedial and arcuate nuclei of the hypothalamus, while females given testosterone at birth express lower estrogen receptor levels in these regions (Kuhnemann et al. 1995). For a more detailed discussion of brain estrogen receptor localization, see Simerly (1990) and Simerly (1993).

Estrogen Receptor Regulation

Estradiol down-regulates the expression of estrogen receptor mRNA levels in a region-specific manner in the adult male brain. For example, gonadectomy (3 days before the assay) up-regulates the relative amount of estrogen receptor mRNA in the male rat medial preoptic nucleus, periventricular preoptic area, and bed nucleus of the stria

terminalis (but not in the encapsulated subdivision of the bed nucleus of the stria terminalis) without changing the total number of estrogen receptor positive cells (Lisciotto and Morrell 1993). Another study showed that estrogen receptor mRNA levels, following gonadectomy (4 days or 2 months), were up-regulated in the preoptic area and bed nucleus of the stria terminalis (Handa et al. 1996). In addition, this increase after gonadectomy was suppressed by estradiol benzoate treatment, but not by dihydrotestosterone treatment.

However, there are conflicting reports of whether or not gonadal steroids regulate estrogen receptor mRNA levels in the medial hypothalamus. Simerly and Young (1991), showed that testosterone down-regulated estrogen receptor mRNA levels in the ventrolateral part of the ventromedial hypothalamus after 3 days of treatment in gonadectomized male rats, but that testosterone did not alter estrogen receptor mRNA levels in the arcuate nucleus. On the other hand, Lauber et al. (1991a), showed that estradiol benzoate did not alter estrogen receptor mRNA levels in gonadectomized male rats within the arcuate nucleus or the ventrolateral aspect of the ventromedial nucleus. This discrepancy could have been because estradiol alone was insufficient to down-regulate the level of estrogen receptor mRNA, without additional androgenic stimulation, in the ventrolateral aspect of the ventromedial nucleus.

Androgen Receptors

The gene encoding the androgen receptor has been cloned from rats (Chang et al. 1988, Tan et al. 1988), mice (Faber et al. 1991), humans (Lubahn et al. 1988), and other animals. It is noteworthy that the androgen receptor is the only steroid receptor found on the X chromosome, and mutations in this gene are detected (in males) more frequently than in any other gene coding for a transcription factor (Semenza 1994). The subject of androgen receptor mutations has been covered in great depth in a recent review (Quigley et al. 1995). The large number of individuals (in both humans, rats, and mice) that have been

studied with single gene mutations resulting in androgen insensitivity indicates that there is probably only 1 gene encoding the androgen receptor. Moreover androgen insensitive animals have served as useful models for studying the actions of androgens (Bullock and Bardin 1977). Other proteins have been identified that can bind androgens, but these have lower affinities and are not members of the nuclear receptor superfamily (Wrogemann et al. 1991, Ramirez 1992).

Androgen receptor mRNA, isolated from the rat ventral prostate and anterior pituitary, is detected by Northern blotting as a single band of 11 kbp. In contrast, androgen receptor mRNA from the rat brain hybridizes to two bands at 11 and 9 kbp, and both of these RNA species are coordinately regulated by androgens (Burgess and Handa 1993). Therefore, it is likely that multiple androgen receptor promoters are used (and/or there is differential mRNA splicing) in the brain; just as multiple promoters are used for estrogen receptor and aromatase mRNA. However, little is known about the functional role of the different androgen receptor mRNAs. If these different mRNAs were translated, heterodimers could form which would alter the function of the androgen receptor complexes (Yen et al. 1997).

Both testosterone and dihydrotestosterone bind to the androgen receptor and induce the proper conformation for receptor transformation and transcriptional activation (Kallio et al. 1994, Zhou et al. 1994b). However, there has been some debate as to whether testosterone or dihydrotestosterone is the more active ligand. Dihydrotestosterone has a higher binding affinity than testosterone in Chinese hamster ovary (CHO) cells transfected with the wild type human androgen receptor (Grino et al. 1990). Dihydrotestosterone is also 10 times more potent than testosterone in inducing mouse mammary tumor virus-chloramphenicol acetlytransferase (MMTV-CAT) reporter gene activity in this system (Deslypere et al. 1992). Additional studies have also reported that in transfected cells (Trapman et al. 1988, Krempelhuber et al. 1994), rat prostate, and rat brain (Barley et al. 1975), androgen receptors have a higher affinity for dihydrotestosterone than testosterone.

However, other studies have reported that androgen receptors have nearly identical binding affinities (~0.1-4 nM) for testosterone and dihydrotestosterone in the rat prostate (Ojasoo et al. 1994) and brain (Naess 1975, Gustafsson et al. 1976, Hannouche et al. 1978, Chamness et al. 1979, Wieland and Fox 1981). Even if there were differences in the androgen receptor's affinity for testosterone and dihydrotestosterone, this does not seem to account for the large differences in the physiological roles of testosterone and dihydrotestosterone. As mentioned earlier, 5α-reduction might prevent testosterone from further metabolism to less androgenic compounds (George 1997).

Androgen Receptor Localization

In rats, androgen receptor immunoreactivity has been detected in the male reproductive tract (prostate, testis, seminal vesicle, coagulating gland, epididymis), female reproductive tract (vagina, cervix, uterus, ovary), brain, kidney, liver, adrenal glands, muscle, pituitary, and other regions as well (Takeda et al. 1990). In the brain, similar androgen receptor-concentrating regions have been found in animals such as frogs (Fischer et al. 1993), goldfish (Pasmanik and Callard 1988), quail (Balthazart et al. 1992), gerbils (Commins and Yahr 1985), hamsters (Wood and Newman 1993a), guinea pigs (Choate and Resko 1992), rats (Simerly et al. 1990), and rhesus monkeys (Clancy et al. 1992). For example, the preoptic area is a brain region that has been found to express androgen receptors in every species examined so far (Everitt and Hokfelt 1990, Lisciotto and Morrell 1994).This indicates that the distribution, and potentially the functions, of brain androgen receptors have been constrained throughout evolution.

The neuroanatomical distribution of the androgen receptor has been studied for over two decades. During this time, techniques with greater specificity and resolution have been developed and applied, although the results for the major androgen receptor-positive regions have not changed substantially. For instance, studies using radioligand binding

assays (Lieberburg et al. 1977, McGinnis et al. 1983, Roselli et al. 1989), immunocytochemistry (Sar et al. 1990, Clancy et al. 1992, Bingaman et al. 1994, Zhou et al. 1994a), and in situ hybridization (Simerly et al. 1990) have all yielded similar results to the initial studies using steroid autoradiography (Sar and Stumpf 1973). In the rat brain, the highest levels of androgen receptors have been found generally in the amygdala, bed nucleus of the stria terminalis, hypothalamus, preoptic area, and septum (McGinnis et al. 1983, Handa et al. 1987, Roselli et al. 1989, Menard and Harlan 1993). The brain regions in male rats expressing androgen receptors are listed in Table 3.

The distribution of androgen receptors in male and female rat brains has been reported to be very similar, even though males express much higher androgen receptor levels than females (Roselli et al. 1989, McGinnis and Katz 1996). However, this lack of a sex difference in the distribution of androgen receptors could be an artifact due to the resolution of the techniques used. For instance, biochemical assays do not have the anatomical resolution provided by techniques such as in situ hybridization. Moreover, sex differences in the connectivity of the afferent and efferent projections of androgen concentrating cells have been reported (Lisciotto and Morrell 1994). Therefore, even though males and females express androgen receptors in similar brain regions, differences in the projections of androgen receptor-positive neurons could be responsible for the behavioral differences of males and females in response to androgens.

Table 3. Brain Regions Expressing Androgen Receptors in the Male Rat

Region	Reference
amygdala	a
arcuate nucleus	b
bed nucleus of the stria terminalis	a
CA-1 region of the hippocampus	b
central tegmental field	c
cortex	b
cranial nerve motor nuclei	d
hypothalamus	a
medial preoptic nucleus	b
septum	a
spinal nucleus of the bulbocavernosis	e
ventral and dorsal horns of the spinal cord	f
ventral premammillary nucleus	b
ventromedial nucleus of the hypothalamus	b

a (McGinnis et al. 1983, Handa et al. 1987, Roselli et al. 1989, Menard and Harlan 1993)
b (Sar et al. 1990)
c (Greco et al. 1994)
d (Sar and Stumpf 1977, Yu and McGinnis 1986, McGinnis and Yu 1995)
e (Matsumoto et al. 1996)
f (Matsuura et al. 1993, Freeman et al. 1995, Lumbroso et al. 1996)

Androgen Receptor Regulation

Quantitative techniques to measure androgen receptor levels in brain tissue have greatly improved since the initial studies involving autoradiographic measurements of systemically injected radiolabeled steroids (Peters and Barrack 1987). Because of its low abundance in brain tissue, measuring small changes in androgen receptor protein and mRNA levels have been troublesome. Northern blot analysis on adult male rat brain (whole) and kidney (Quarmby et al. 1990), and adult male rat anterior pituitary, preoptic area, medial basal hypothalamus, and hippocampus (Burgess and Handa 1993) indicated that steady state androgen receptor mRNA levels were elevated after gonadectomy (4 days and 7 weeks, respectively). Administration of dihydrotestosterone for 1 day (to males that were gonadectomized 4 days prior) decreased androgen receptor mRNA levels in the anterior pituitary and preoptic area-hypothalamus, but treatment with estradiol benzoate did

not alter the concentration of androgen receptor mRNA in either tissue (Burgess and Handa 1993).

Application of ribonuclease protection-solution hybridization (RNase protection) assays (Burgess and Handa 1993) and reverse transcription-polymerase chain reaction (RT-PCR) assays (Osada et al. 1993) have proven to be useful tools in quantitating the low levels of androgen receptor mRNA from individual brain regions. Use of these techniques revealed brain region-specific differences in the hormonal regulation of androgen receptor mRNA levels. For instance, 4 days after gonadectomy, the level of androgen receptor mRNA in the hippocampus was decreased (Kerr et al. 1995), but the level of androgen receptor mRNA in the preoptic area-hypothalamus was increased (Burgess and Handa 1993). Moreover, 4 days after gonadectomy, the level of androgen receptor mRNA in the amygdala was unchanged. This result was very interesting because in the amygdala, aromatase mRNA levels were also not altered after gonadectomy (discussed on page 14).

Not only were there region-specific differences in the hormonal regulation of androgen receptor mRNA levels, but the time following gonadectomy was important as well, because androgen receptor mRNA levels in rat brain tissue differed depending on the length of time after gonadectomy. For example, androgen receptor mRNA levels were significantly increased 4 days after gonadectomy, but androgen receptor mRNA levels were significantly decreased 2 months after gonadectomy in both the preoptic area and the bed nucleus of the stria terminalis (Handa et al. 1996). Moreover, while a single injection (24 hours before the assay) of estradiol benzoate had no effect on androgen receptor mRNA levels in gonadectomized males just 4 days after gonadectomy, 2 months after gonadectomy, estradiol-filled Silastic capsules (implanted 2 weeks before the assay) significantly increased androgen receptor mRNA levels (Handa et al. 1996). Therefore, this difference in the hormonal regulation of androgen receptor mRNA levels, 4 days and 2 months after gonadectomy, implied that other mechanisms were involved, besides simple transcriptional regulation of androgen receptor mRNA levels.

Androgen receptor protein levels were also regulated in a complex manner. For example, even though androgen receptor mRNA levels were elevated 4 days after gonadectomy (in the preoptic area and bed nucleus of the stria terminalis), the levels of androgen receptor immunoreactivity were decreased relative to intact males (Handa et al. 1996). However, this discrepancy could have been due to an experimental artifact in the data. For instance, the immunocytochemical signal generated by cytoplasmic androgen receptors, due to its diffuse nature, might not have been directly comparable to the intense nuclear staining found in intact males. This could have lead to the interpretation that the protein levels were decreased; when in fact, they were not (Wood and Newman 1993a). On the other hand, androgen receptors were found to be unstable in the absence of androgens in cultured cells (Zhou et al. 1994b). Therefore, besides simple transcriptional regulation, other mechanisms (such as the posttranslational control of androgen receptor protein levels) are likely to occur (Sheridan 1983, Liao et al. 1989), although their role in brain androgen receptor action is unclear.

Brain Colocalization of Aromatase, Estrogen Receptors, and Androgen Receptors

Aromatase, estrogen receptors, and androgen receptors are all generally found within the same brain regions in male and female rats (reviewed in Stumpf and Sar 1976, McEwen 1980). For example, in situ hybridization studies on the distribution of androgen and estrogen receptor mRNA have indicated that these messages have a similar pattern of expression (Simerly et al. 1990). Furthermore, aromatase activity (Roselli and Resko 1987) and aromatase mRNA (Abdelgadir et al. 1994, Wagner and Morrell 1996) have been localized in adult male rats to areas that also express estrogen and androgen receptors. These areas include the medial preoptic area, ventromedial hypothalamus, and amygdala. The proximity of these three proteins within the brain could allow for interactions that

would modulate the hormonal activation of neural activity. For instance, testosterone acting through the androgen receptor could stimulate the transcription of aromatase. This would regulate the levels of both estradiol (by its synthesis) and testosterone (by its catabolism). However, it is not known whether the effects of testosterone on aromatase (mRNA or activity levels) are due to the direct action of androgen receptors within the cells expressing aromatase. Moreover, it is not known if estradiol, formed by the aromatization of testosterone, acts within the cells where it was produced and/or if the estradiol influences other cells in a paracrine fashion.

There is not a large body of evidence indicating that there is cellular colocalization of these proteins, especially in rats. Some of the most detailed studies on the cellular colocalization of androgen and estrogen receptor immunoreactivity have been conducted in the male hamster brain (Wood et al. 1992, Wood and Newman 1995a). This work has shown that androgen and estrogen receptors are often, but not always, coexpressed within the same cells in the male hamster medial preoptic area, bed nucleus of the stria terminals, ventromedial nucleus of the hypothalamus, and the amygdalohippocampal area. In the rat brain, Diano et al. (1997) found that androgen and estrogen receptors did colocalize in the lateral septum (except for the dorsal division that does not express estrogen receptors), periventricular areas of the hypothalamus, anterior hypothalamus, arcuate nucleus, and medial amygdala. However, this study was not as detailed as the work carried out in hamsters, and more work is needed to determine the extent of colocalization of androgen and estrogen receptors in the male rat brain.

Nonclassical Mechanisms of Steroid Hormone Action

Genes that are regulated by steroid hormones are said to be "hormone-dependent." However, this does not necessarily mean that all hormone-dependent genes are transcriptionally regulated by the specific interactions of steroid receptors on the genes'

hormone response element(s). Besides regulating transcription by activating nuclear receptor proteins, steroid hormones can have direct effects on membrane fluidity and/or bind to sites located within the plasma membrane. This is in contrast to the dogma in many textbooks (Darnell et al. 1990, Lehninger et al. 1993, Alberts 1994, Stryer 1995), which states that steroid hormones exert their effects transcriptionally via nuclear steroid receptors (Wehling 1995, Ramirez 1996). Some of these other mechanisms have also been called "nongenomic actions of steroids." However, the term "nongenomic" can be misleading, because it implies that genomic action is not involved (Lenard and Montminy 1991). Therefore, the phrase "nonclassical mechanisms of steroid hormone action" will be used instead. This rapidly expanding field has been the subject of many comprehensive reviews (Orchinik and McEwen 1993, Brann et al. 1995, Olsen and Sapp 1995, Lambert et al. 1996).

Support for the hypothesis that steroid hormones can have nonclassical mechanisms has come from studies showing that there are some very rapid effects (within a few minutes) of steroid hormones (Brann et al. 1995). For example, estradiol treatment rapidly (within 15 minutes) induces the phosphorylation of the cAMP response element binding protein (CREB) in the preoptic area and bed nucleus of the stria terminalis of adult female rats (Zhou et al. 1996). This also indicates that estradiol could influence the transcription of genes that do not contain estrogen response elements through the activation of CREB. In addition to using rapid and short acting effects as the criteria for assessing the nonclassical actions of steroid hormones, the use of protein-steroid conjugates, which do not pass through the plasma membrane, have served as powerful tools (Zheng et al. 1996). For example, protein-steroid conjugates have been used to characterize membrane binding of steroid hormones, and to show that the nonclassical effects of steroids can occur without steroid entry into cells (Gorczynska and Handelsman 1995, Fiorelli et al. 1996). The specific binding of radiolabeled-corticosterone, -estradiol, -testosterone, and -progesterone have all been shown to occur in brain synaptic plasma membranes, with dissociation

constants around 10^{-8} M (reviewed in Towle and Sze 1983, Ramirez and Zheng 1996). Moreover, a number of well-known membrane proteins have been found to interact with steroid hormones: including oxytocin receptors (Caldwell et al. 1994), NMDA receptors (McEwen 1996), opioid receptors (Schwarz and Pohl 1994), and $GABA_A$ receptors (Gee et al. 1995, Lambert et al. 1995, Olsen and Sapp 1995, Mahesh et al. 1996, Rupprecht et al. 1996). However, the physiological relevance of the interactions between gonadal steroid hormones and these membrane proteins is not well understood.

Along with nonclassical mechanisms of steroid hormone signaling within individual cells, steroid hormones can also influence distal cells by the modulation of neuronal activity (Smith et al. 1987). For example, the androgenic regulation of gonadotropin releasing hormone (GnRH) release is an area where the effects of testosterone are thought to occur at a distal site (Sagrillo et al. 1996). Androgens are known to inhibit the release of GnRH from cells in the hypothalamus, that in turn, inhibit the pituitary's secretion of gonadotropins. Studies using androgen antagonists (Roselli and Resko 1984, Grattan et al. 1996) and androgen-insensitive (Tfm) strains of rats (Naess et al. 1976) have shown that GnRH action is regulated by androgen receptors. However, androgen receptors do not colocalize with GnRH immunopositive neurons in the male rat hypothalamus (Huang and Harlan 1993). Therefore, testosterone, acting through the androgen receptor, might regulate the release of GnRH through a trans-synaptic mechanism (Ginty et al. 1992).

In summary, steroid hormone signaling is much more complex than the simple model of nuclear receptor action presented in Figure 3 (Kawata 1995). However, there is still a considerable body of research supporting a classical mechanism of steroid hormone action in mediating hormone-dependent behaviors (McEwen 1980, McEwen 1988). This is best exemplified by the role of estrogen receptors in facilitating female copulatory behavior (McEwen et al. 1987) and androgen receptors in facilitating male copulatory behavior (McGinnis and Mirth 1986, McGinnis and Dreifuss 1989). Consequently, the roles of cell

nuclear androgen and estrogen receptors in the hormonal regulation of hormone-dependent sociosexual behaviors in male rats are a focal point for this project.

Effects of Gonadal Steroid Hormones on the Brain & Behavior

The effects of gonadal steroid hormones can be divided into two broad classes (Arnold and Breedlove 1985). First, there are organizational effects of gonadal steroid hormones on developing tissue sensitive to steroid hormones during critical periods. Second, there are activational effects of steroid hormones on adult tissue (Balthazart and Schumacher 1984, McGinnis et al. 1989). A critical period is defined here as a window of time where tissues are sensitive to permanent modification (i.e., organization) by steroid hormones. The activational effects of steroid hormones do not result in permanent cellular changes and are reversible in the absence of hormone. Activational effects of steroid hormones are believed to change the way the adult brain functions by altering steroid-dependent behavioral circuits (Lisk 1967, Greenstein 1984, Huang et al. 1989, Kaplan and McGinnis 1989, Rose 1990, Resko et al. 1993, Wood and Newman 1993b). This results in the presentation of distinct behavioral patterns, which are dependent upon the animal's hormonal milieu (Feder 1978).

The role of steroid hormones on sexually dimorphic development is an example of an organizational effect (Feder 1981, Arnold and Gorski 1984, McCarthy 1994). As early as 1922, Lille hypothesized that hormones modified sexual "structure, function, and psyche" during development (reviewed in Aberle and Corner 1953). This hypothesis, posed for the National Research Council for Research in Problems of Sex, provided a major impetus in establishing laboratory funding for this emerging field and was inspired by Lille's work with domestic cows known as free-martins (Lille 1917). Free-martins are the female twins of male calves who are defeminized by steroid hormones secreted from their brothers in utero. Other work showed that sex differences in gonadotrophic function were determined by a testis-borne factor secreted during a critical period in early postnatal development in male rats (Pfeiffer 1935). Unfortunately, the authors of that study

mistakenly thought that it was the pituitary, and not the hypothalamus, that was sexually differentiated.

The foundation for the hypothesis that steroid hormones can organize brain sexual differentiation came from a landmark study (Phoenix et al. 1959) showing that injections of testosterone into pregnant guinea pigs, during specified periods, resulted in masculinization of the female offspring (i.e., they had male external genitalia and exhibited male-like copulatory behavior when injected with testosterone in adulthood) with concurrent defeminization (i.e., they were behaviorally insensitive to injections of estradiol and progesterone in adulthood). This observation was later validated in birds (Balthazart 1989, Panzica et al. 1996) and other rodents (McEwen et al. 1977, Hutchison et al. 1995). Besides studies showing that gonadal steroids organize functional aspects of brain sexual differentiation, a number of sex differences in brain morphology have been published (Arnold and Gorski 1984, Hines et al. 1992, Davis et al. 1995). The best example of this is the sexually dimorphic nucleus of the preoptic area in rodents (SDN-POA, Gorski et al. 1980). Studies on the hormonal organization of sexually dimorphic brain structure and function have given support for the hypothesis that steroid hormones, rather than genetic sex (although genetic sex determines which steroid hormones are produced), are the primary determinant of brain sexual differentiation (Goy and McEwen 1980, Baum 1990).

Berthold's experiment, observing the behavioral and physical effects of removing and then transplanting the testes from roosters (described at the beginning of this chapter), is a classic example of the activational effects of steroid hormones (reviewed in Forbes 1949). However, the neural mechanisms mediating the hormone-dependent behaviors Berthold observed were previously organized by steroid hormones during the rooster's development. Interestingly, there are no known activational effects of steroid hormones on the adult brain that are independent of the organization of steroid-dependent neural circuits during development (Sheridan et al. 1974, Swerdloff et al. 1992, Arnold and Schlinger 1993). On the other hand, the display of some sexually dimorphic behaviors that are

organized by steroid hormones, such as play fighting in juvenile rats, occur regardless of whether steroid hormones are present or absent (Beatty 1992). The work described hereafter will focus on the activational role of steroid hormones in adult male rats that have undergone "normal" development (i.e., without experimental manipulations prior to adulthood).

Hormone-Dependent Sociosexual Behaviors

The systematic study of hormonal factors mediating animal behavior matured into the discipline of behavioral endocrinology in the period between 1900 and 1950 (reviewed in Beach 1981). During this time, techniques were established for the study of male copulatory behavior, which are still used today (Beach 1938b), and the roles of testis-borne substances in regulating male copulatory behavior were refined (reviewed in Beach 1974). During this period, two major contributions were that individual components of male copulatory behavior could be observed as discrete parameters, and that individual aspects of copulatory performance were differentially influenced by gonadectomy in adult male rats (Stone 1923). For instance, the withdrawal of testicular hormones was shown to have a gradual inhibitory effect on male copulatory behavior, where the animals first stopped ejaculating, later they stopped intromitting, and then after several weeks they stopped mounting(Stone 1927). Stone (1923) also showed that there were notable interanimal behavioral differences following endocrine manipulations. Other work showed that the "sexual drive" of male rats, determined by observing the males cross an electrified grid to reach sexually-receptive females (using the Columbia Obstruction Apparatus), also decreased gradually over several weeks after gonadectomy (Nissen 1929).

The outcome of this early work was that many behaviors were found to be regulated in a steroid hormone-dependent manner (i.e., steroid hormones were necessary to activate the neural circuits in the brain that mediated these behaviors in adult animals,

reviewed in Beach 1974). Examples of hormone-dependent sociosexual behaviors displayed by male and female rats are listed in Table 4. However, rats have not been the only experimental subjects in behavioral endocrinology. It should be noted that the hormonal regulation of many sociosexual behaviors have also been actively studied in other taxa such as hamsters (Wood and Newman 1993b, Wood and Newman 1995b), ferrets (Carroll et al. 1988, Baum et al. 1990), gerbils (Yahr et al. 1979, Yahr et al. 1994), mice (Levine et al. 1965, Vale and Ray 1972, Chubb 1987), cynomolgus monkeys (Zumpe and Michael 1985), rhesus monkeys (Michael et al. 1990), Japanese quail (Balthazart et al. 1990, Balthazart et al. 1995, Panzica et al. 1996), and numerous others. The fact that the hormonal regulation of behavior has been studied in so many different kinds of animals has been attributed to the work of Beach (1950), because of his appreciation for the comparative approach to behavioral analysis (reviewed in Beach 1976a, Drewsbury 1978).

Finally, it should be noted that the effects of hormones on behavior are not solely determined by the hormone's chemical structure. Other factors such as the species, sex, age, prior experiences, and the animal's environment all play a role in determining behavioral responses (Beach 1948b, Adler 1978, Diamond et al. 1996). In general, hormones by themselves are not sufficient to elicit behavioral responses in the absence of species- and context-specific cues. For example, Beach (1942a) showed that the stimuli leading to male copulatory behavior were the result of two joint variables: the male's

Table 4. Examples of Hormone-Dependent Sociosexual Behaviors

Behavior	Reviewed in:
aggressive behavior	(Barfield 1984, Knol and Egberink-Alink 1989)
female copulatory behavior	(Komisaruk 1978, McEwen et al. 1987, Erskine 1989)
male copulatory behavior	(Beach 1947b, Beach 1967, Sachs and Barfield 1974, Davidson 1977)
maternal behavior	(Modney and Hatton 1994, Rosenblatt et al. 1994)
partner preference	(Everitt 1990, Schechter and Calcagnetti 1993)
scent marking	(Lee et al. 1984, Matochik and Barfield 1991)
ultrasonic vocalizations	(Barfield et al. 1979, Barfield and Thomas 1986, White and Barfield 1987)

responsiveness and the ability of the female to provide an incentive stimulus. Proceptive behaviors (hopping/darting and ear wiggling) displayed by sexually receptive females are examples of incentive stimuli for male rats (Schein and Hale 1965, Erskine 1989). Moreover, no single sensory modality exclusively mediates the activation of copulatory behavior in rats (Beach 1967), but the loss of 1 or more sensory modalities reduces sexual excitability (Beach 1942b). So, a complex pattern of multisensory integration is necessary for male rats to interpret behavioral input from their environment in order to produce appropriate behavioral responses (Stern 1990). This complex process of integrating the hormonal and sensory information necessary for the display of copulatory behavior led Beach (1948b) to postulate that this was a source of the variability observed in measuring hormone-dependent sociosexual behaviors.

Male Copulatory Behavior in Rats

Of the many hormone-dependent sociosexual behaviors in male rats, the neural basis of male copulatory behavior has been best characterized (Malsbury and Pfaff 1974, Sachs and Barfield 1974, Davidson 1975). First, some definitions need to be made as to what is meant by the phrase "male copulatory behavior." There is a necessity for precise definitions in behavioral science because identical terms often mean very different things to different authors (especially when interspecies comparisons are made, Beach 1965, 1967). The phrase "male sexual behavior" is commonly used to describe male-typical patterns of mating behavior. However, the word "sexual" in this context is somewhat ambiguous, and the more literal term "copulatory" (defined in the American Heritage Dictionary as to engage in coitus or sexual intercourse) will be used instead.

The general phrase "male copulatory behavior" does not refer to a single behavior per se, but to a complex pattern of different behaviors consisting of the following behavioral segments: exploratory behavior (genital sniffing of a female), precopulatory

50 kHz ultrasonic vocalizations, mounting the female with pelvic thrusting, intromissions (mounting with penile insertion), ejaculation, postcopulatory 22 kHz ultrasonic vocalizations, and genital grooming. All the segments are repeated until the male reaches sexual exhaustion or the female leaves (Sachs and Barfield 1970). The stereotyped behavioral pattern (having low variability within individual species) in rodent copulatory behavior has prompted extensive studies into its neural basis (Sachs and Barfield 1970). However, even among different species of muroid rodents, there is remarkable diversity in copulatory behavior (Drewsbury 1975). A quotation from Darwin (1871 p. 577) highlights this conundrum: "The courtship of animals is by no means so simple and short an affair as might be thought."

During coitus, ejaculation is an aspect of copulatory behavior that is distinct from all the other components, such as mounting and intromitting. This is because the fundamental objective of copulation is successful procreation (at least from a sociobiological point of view), and for that to occur, the transmission of genetic information from the male to the female during ejaculation is necessary. Therefore, male copulatory behavior can be seen as a goal-directed behavior culminating with ejaculation. On the other hand, ejaculation does not necessarily guarantee fertilization, and other components of copulatory behavior in both male and females have been said to facilitate conception (Beach 1947a, Etkin 1964, Caspari 1965, Yang and Clemens 1997). A few examples of these behaviors in male rats are the repetitive nature of intromitting prior to ejaculating and the males' postejaculatory refractory period (Sachs and Barfield 1974); and for female rats, the pacing of copulatory behavior (Krieger and Barfield 1976, Mendelson and Gorzalka 1987, Erskine 1989). Therefore, observations of copulatory behavior usually consist of measuring many different parameters related to mating (such as the frequencies and latencies of mounts, intromissions, and ejaculations), rather than just whether or not the male ejaculated.

Female Copulatory Behavior in Rats

Female rats are not idle during mating. Sexually receptive female rats exhibit proceptive (solicitation) behaviors such as hopping/darting, orientating themselves away from the males, and ear-wiggling in response to male rats or tactile stimuli. These proceptive behaviors play an important role in stimulating male rats to copulate (Madlafousek et al. 1976). When a male mounts a sexually receptive female, she arches her back and moves her tail laterally in a stereotyped posture called lordosis. Nonreceptive females fight with males who try to copulate with them. For reviews of female copulatory behavior in rodents, see Beach (1976b), Komisaruk (1978), and Morali and Beyer (1979).

One example of a difference between male and female copulatory behavior in rats is that female copulatory behavior varies throughout the estrus cycle (peaking on the day of estrus), but the level of male copulatory behavior is relatively constant (correlating with the tonic presence of serum testosterone). Just 6 hours of estradiol exposure is sufficient for the facilitation of lordosis 24 hours later in female rats given progesterone (Rainbow et al. 1982a). However, in male rats a minimum of 21 hours of testosterone exposure per day, for at least 10 days, is necessary for restoration of male copulatory behavior (McGinnis et al. 1989). This indicates that the neural substrates for male and female copulatory behavior could be distinct from each other (Pfaff 1970, Fadem and Barfield 1982, Moreines et al. 1986, McEwen et al. 1987, Lauber et al. 1991c). Another significant difference between male and female copulatory behavior is that virgin female rats instinctively exhibit high levels of receptivity and proceptivity under the appropriate hormonal and environmental stimuli, while virgin male rats need to learn how to copulate effectively over repeated trials (Beach 1942b, Beach 1948a).

Motivation and Performance

Components of hormone-dependent sociosexual behaviors relating to copulation are often subdivided into motivational (also known as appetitive or anticipatory behaviors) and performance (also known as consumatory behaviors) aspects (Beach 1956, Sachs and Meisel 1988, Everitt 1990). For example, during copulation different behavioral components have been partitioned into motivational aspects (such mount latency, and post ejaculatory interval) or performance aspects (number of intromissions relative to the number of mounts or ejaculation latency). Unfortunately, categorizing individual parameters of male copulatory behavior (such as mount and intromission frequencies/latencies) as being part of either motivational or performance aspects is somewhat subjective (Beach 1956). The application of factor analysis on the individual parameters of male copulatory behavior has proven to be a useful tool for making this process more objective (Sachs and Barfield 1974, Drewsbury 1979, Pfaus et al. 1990). Factor analyses in two separate studies, involving different testing paradigms, have produced very similar results, where much of the intersubject variance was attributed to the following three factors: initiation, copulatory rate, and hit rate (Sachs and Barfield 1974, Pfaus et al. 1990). Conceptually these three factors can be partitioned into motivational (initiation) and performance (copulatory rate and hit rate) aspects.

Besides direct observations of male copulatory behavior, other tests have been developed to infer sexual motivation. Nissen's (1929) and Moss's (1924) early work with the Columbia Obstruction Apparatus, and Everitt's more recent work measuring appetitive aspects of copulatory behavior with second order schedules of sexual reinforcement (Everitt et al. 1987, Everitt and Stacey 1987) are examples of efforts which have been made to quantify sexual drive. Another test to infer sexual motivation is the partner preference test (Edwards and Einhorn 1986). The basis of this test is that intact (or testosterone-treated gonadectomized males) male rats prefer to spend more time with sexually receptive females

than they do with nonreceptive females or to be alone, even if the males are prevented from intromitting. Therefore, the partner preference test is useful for measuring sexual motivation in a way that is independent of coitus.

Other Behaviors Closely Associated with Mating

There are other hormone-dependent sociosexual behaviors that are related to copulatory behavior, but not distinctly involved in mating per se. Examples of two of these social behaviors, which occur in both males and females, are scent marking and ultrasonic vocalizations (McIntosh et al. 1979). Nevertheless, much less attention is paid to these other behaviors, relative to copulatory behavior in rats. In male rats, both scent marking and ultrasonic vocalizations decline after gonadectomy, and they are restored by testosterone treatment (Parrott and Barfield 1975, Matochik and Barfield 1991). Scent marking also declines in male gerbils after gonadectomy, and can be restored with testosterone treatment (Yahr et al. 1979, Yahr et al. 1980). Scent marking serves as a way for animals to mark territory by providing olfactory cues and has been described as a motivated behavior (Birke and Sadler 1983, Lee et al. 1984, Takahashi 1990). This can be measured by observing the frequency of sebaceous scent marking of novel objects by male rats in response to olfactory cues from sexually receptive female rats (Matochik and Barfield 1991). Ultrasonic vocalizations are another way in which rats communicate (Barfield et al. 1979). These vocalizations generally occur in two categories: First, male and female rats make 50 kHz calls to each other in the form of rapid chirps, which have been correlated with sexual motivation and the facilitation of copulatory behavior (White and Barfield 1990). Second, just after males ejaculate they start to make long calls centering around 22 kHz (Barfield and Geyer 1972). These long lower frequency calls have been attributed to coordinating the male's postejaculatory refractory period with the activity of the female (Barfield and Geyer 1975, Parrott and Barfield 1975). Therefore, scent marking

and ultrasonic vocalizations have been shown to play important roles in the coordination of copulatory behavior between male and female rats (Barfield and Thomas 1986, White and Barfield 1987).

Neuroanatomical Basis of Male Copulatory Behavior

Several brain regions have also been conceptually grouped together for their role in mediating motivational and performance aspects of male copulatory behavior. For example, the medial preoptic area has been associated with "performance aspects" such as mounts, intromissions, and ejaculations (Sachs and Meisel 1988); while the amygdala and ventral striatum have been associated with "motivational or arousal aspects" having to do with preparatory and precopulatory behaviors (Everitt 1990). However, this is probably a gross oversimplification of the roles these individual brain regions really play in mediating male copulatory behavior (Baum 1995). The areas often found to modulate copulatory behavior are typically located in phylogenetically older structures (reviewed in de Groot 1965, Barfield 1979), such as those within the limbic system. The limbic system of the brain mediates the processing of memory, motivation, emotion, and many autonomic functions (Riss et al. 1969). Many limbic brain regions (amygdala, anterior thalamus, hippocampus, hypothalamus, and septum) are reciprocally interconnected by prominent fiber projections, such as the stria terminalis and fornix shown in Figure 6 (Simerly 1995, Parent 1996). Therefore, the limbic system has the potential to function as a network involved in the complex task of mediating male copulatory behavior (de Groot 1965, Simerly and Swanson 1986, Robbins et al. 1989, Everitt and Hokfelt 1990, Everitt et al. 1991).

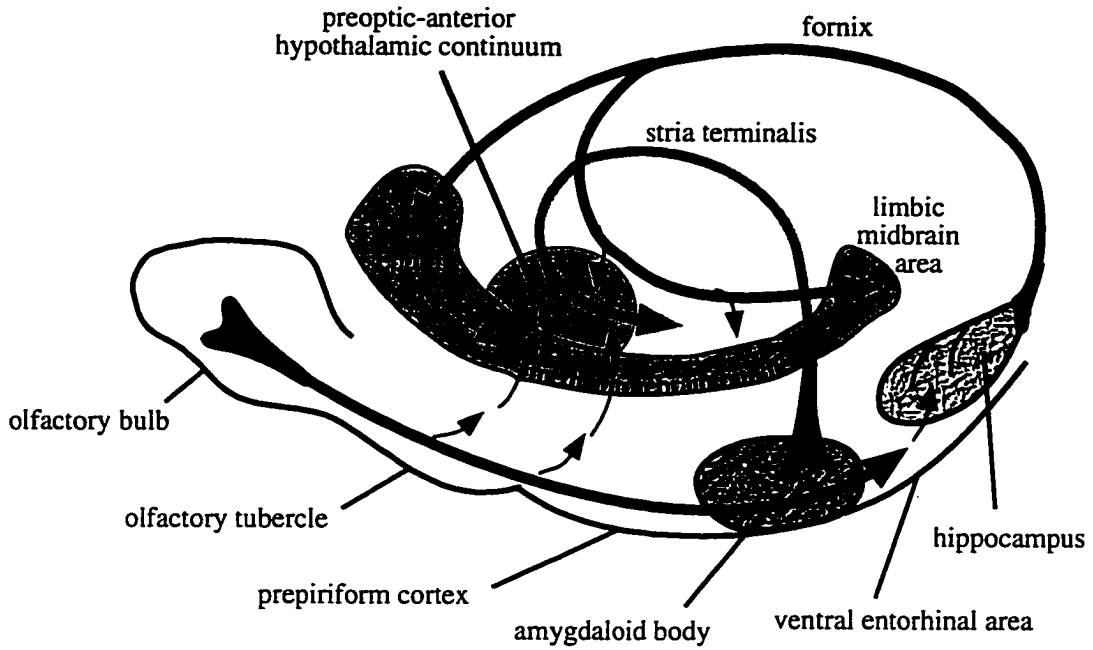


Figure 6. Afferent Projections into the Preoptic-Anterior Hypothalamic Continuum

Reprinted from the Brain Research, Volume 3, Heimer, L. and Larsson, K. Impairment of mating behavior in male rats following lesions in the preoptic-anterior hypothalamic continuum. Pages 248-263. Copyright 1966, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

Beach (1940, 1942a) emphasized that sensory stimuli influenced all levels of the central nervous system, and that sensory stimuli did not elicit copulatory behavior by activating a simple mating reflex arc in the brain (reviewed in Rosenblatt 1978). However, this did not mean that reflexes were not involved in mediating aspects of copulatory behavior. Beach was specifically referring to the idea of a single "mating reflex" with a discrete localization in the cerebral cortex. This model fit well with the theory of Beach's mentor, K. S. Lashley, who suggested that all regions of the cerebral cortex participated in the cortical control of behavior (the mass-action hypothesis, Lashley 1933). More recent studies have supported the hypothesis that distributed cortical function mediates male copulatory behavior in rats, by monitoring the progressive recovery of male copulatory

behavior following ablation of the cingulate cortex (Agmo et al. 1995). In addition, central nervous stimulants, which did not influence male copulatory behavior in control rats (without lesions), facilitated the recovery of male copulatory behavior following ablation of the cingulate cortex (Agmo and Villalpando 1995). These data indicated that generalized cortical function plays a role in the initiation of male copulatory behavior. One of Beach's major contributions to the field of behavioral endocrinology was the concept that lower-level neural functions, such as spinal and myelencephalic control of reflexes involved in copulatory behavior, could be facilitated or inhibited by higher-levels of neural organization from more rostral brain regions (Beach 1967, Hart 1978). This concept is exemplified by the role of the medial preoptic area in overriding the tonic inhibition of neurons located in the nucleus paragigantocellularis, which mediates the urethrogenital reflex in male rats, to facilitate ejaculation (Marson and McKenna 1994).

While an extreme localizationist view of behavior could be seen as misguided (in the sense of attributing individual behaviors to discrete brain regions), experimental lesion studies have shown that the ablation of a number of other brain regions outside of the neocortex resulted in the permanent loss of male copulatory behavior. For example, many lesion studies have consistently cited the importance of the medial preoptic area in the modulation of male copulatory behavior (see Table 5). Lesions in the medial preoptic area of adult male rats lead to a complete loss of male copulatory behavior without any recovery of function. Although medial preoptic area lesions in adult male rats are believed to block male copulatory behavior permanently, males which had prepuberal lesions in the preoptic area displayed normal male copulatory behavior as adults (Twiggs et al. 1978, Meisel 1983). This reinforces the notion that the initial establishment of behavioral patterns (and the corresponding neural patterns) after brain injury is distinct from the recovery of function in preestablished neural circuits (such as in males who were already copulating at the time of the lesion).

Table 5. Studies Showing that Preoptic Area Lesions Inhibit Male Copulatory Behavior in Adult Rats

Type of Lesion	Reference
electrolytic	(Larsson and Heimer 1964)
electrolytic	(Heimer and Larsson 1966)
electrolytic	(Christensen et al. 1977)
electrolytic	(Ginton and Merari 1977)
electrolytic	(Kamel and Frankel 1978)
electrolytic	(Ryan and Frankel 1978)
electrolytic	(Van De Poll and Van Dis 1979)
electrolytic	(Bermond 1982)
electrolytic, neurotoxic (ibotenic acid)	(Hansen et al. 1982)
electrolytic	(Arendash and Gorski 1983)
electrolytic	(Meisel 1983)
electrolytic	(Barckett and Edwards 1984)
electrolytic	(Edwards and Einhorn 1986)
electrolytic	(Everitt and Stacey 1987)
electrolytic	(Hlinak et al. 1987)
dopaminergic (6-OHDA)	(Sirinathsinghji 1987)
electrolytic	(De Jonge et al. 1989)
electrolytic	(Kondo et al. 1990)
dopaminergic (MPTP)	(Bazzett et al. 1992)
electrolytic	(Paredes et al. 1993a)
excitotoxic (NMDA)	(Edwards et al. 1996)

Figure 6 displays the spatial relationship between the medial preoptic area, the surrounding brain regions, and the projections that interconnect these areas. A good example of the specificity in the role of medial preoptic neurons in mediating male copulatory behavior comes from a study where fetal hypothalamic transplants, but not fetal cortical transplants, into the medial preoptic area were able to restore male copulatory behavior in adult male rats with medial preoptic area lesions (Paredes et al. 1990b, Paredes et al. 1993b). Moreover, preoptic area lesions inhibit not only male copulatory behavior, but also partner preference in adult male ferrets (Paredes and Baum 1995, Kindon et al.

1996), and scent marking in adult male gerbils (Commins and Yahr 1984, Yahr and Stephens 1987).

Other evidence for the role of the medial preoptic area in mediating male copulatory behavior has come from electrophysiological experiments. Multiple unit activities recorded from freely-moving male rats indicated that the electrophysiological pattern within the medial preoptic area changed during copulation (Horio et al. 1986), and that repeated electrical stimulation (kindling) in the medial preoptic area, but not in the amygdala, facilitated copulatory behavior in inactive male rats (Paredes et al. 1990a). Lesions just caudal to the preoptic area throughout the hypothalamus have had varied effects on male rat copulatory behavior. For instance, lesions within the anterior hypothalamus inhibited male copulatory behavior (Van De Poll and Van Dis 1979), but ablation of the ventromedial hypothalamus might have actually facilitated both male copulatory behavior and female copulatory behavior in male rats (Christensen et al. 1977). Lesions to the lateral hypothalamus and zona incerta also reduced male copulatory behavior (Maillard and Edwards 1991).

The median forebrain bundle contains efferents from the medial preoptic area to several brainstem nuclei (dorsolateral tegmentum, central gray, and the A9 and A10 noradrenergic cell groups). Therefore, these brainstem nuclei receive direct neural input from the medial preoptic area (Simerly and Swanson 1986, Simerly and Swanson 1988). Lesions within the median forebrain bundle (Hitt et al. 1970, Hitt et al. 1973, Caggiula et al. 1975) or the dorsolateral tegmentum (Barckett and Edwards 1984) completely disrupt male copulatory behavior, while lesions within the A9 cell group only impair mating, and lesions within the A10 cell group just increase the postejaculatory interval. Interestingly, central gray lesions might actually facilitate male copulatory behavior (Brackett et al. 1986). Other studies show that rostral midbrain lesions also result in the reduction of the postejaculatory interval and therefore facilitate male copulatory behavior (Barfield et al. 1975, Clark et al. 1975). In addition, it is important to note that the loss of copulatory

activity following lesions in the medial forebrain bundle is not due to pituitary-gonadal dysfunction, because androgen treatment does not restore copulatory behavior in animals with these lesions (Caggiula et al. 1975).

Along with the preoptic area-hypothalamic continuum and the brainstem, the amygdala and its major efferent projection, the stria terminalis (see Figure 6), are also important for mediating male copulatory behavior (Kostarczyk 1986). Within the subdivisions of the amygdala, medial amygdala lesions result in severe copulatory deficits, cortical amygdala lesions impair intromission and ejaculatory responses, and basolateral amygdala lesions have no effect (Bermond 1982, Kondo 1992). Transection of the stria terminalis (which reciprocally connects the medial preoptic area and the amygdala, Giantonio et al. 1970, Bermond 1982) and lesions of the bed nucleus of the stria terminalis (an androgen receptor-rich nucleus at the end of the strial terminalis near the preoptic area, Emery and Sachs 1976, Claro et al. 1995) both decrease male copulatory behavior in adult rats. Moreover, an unidentified nonstrial pathway from the amygdala may influence male rat copulatory behavior (Kondo and Yamanouchi 1995). Also, experimental lesions do not influence male copulatory behavior in every limbic brain region. For instance, septal nuclei lesions do not influence male copulatory behavior (McGinnis and Gorski 1980, Becker et al. 1992, Kondo 1992).

The results from amygdala lesion studies on male copulatory behavior fit in well with the neuroanatomical organization of the amygdala and its connections. Both the cortical and medial regions of the amygdala project to the medial preoptic area and the anterior hypothalamus by way of the stria terminalis, and both of these regions also receive direct input from the olfactory bulbs (Alheid et al. 1995). However, only the medial amygdala receives projections from the accessory olfactory bulbs (Ichikawa 1987). This is in contrast to the basolateral amygdala, which does not receive afferents from either the olfactory bulbs or the accessory olfactory bulbs, and it does not send projections into the stria terminalis. Therefore, it is likely that the amygdala plays an important role in the

processing of olfactory information relevant for copulatory behavior because the sites that mediate male copulatory behavior within the amygdala receive heavy olfactory input (Riss et al. 1969) and also share reciprocal projections with the preoptic area and hypothalamus.

Additional evidence for the hypothesis that the amygdala plays a role in processing olfactory information has come from work on olfactory bulbectomized adult male rats. Removal of the olfactory bulbs of adult male rats permanently disrupts male copulatory behavior, and this effect is not due to either secondary androgen insufficiency or to the loss of the sense of smell (reviewed in Meisel et al. 1984). The ablation of the olfactory bulbs in these animals results in a decrease in cell nuclear androgen receptor occupation within the amygdala and hypothalamus (Lumia et al. 1987). Since the decrease in male copulatory behavior is associated with the reduction in cell nuclear androgen receptor occupation, this work indicates that the modulation of the neural connections between the olfactory bulbs and androgen receptor-rich regions within the hypothalamus and amygdala are important for male rat copulatory behavior. On the other hand, olfactory bulbectomized adult female rats actually have higher levels of sexual behavior, and this has been associated with increases in amygdala cell nuclear estrogen receptor occupation (McGinnis et al. 1985).

Further evidence for the role of androgens in modulating limbic function comes from electrophysiological experiments. In the medial preoptic area, testosterone can change the absolute refractory period measured in afferents from, and efferents to, the median forebrain bundle. However, this study also shows that the projections from the medial preoptic area to the lateral septum are not effected by testosterone (Kendrick 1983). In addition, gonadectomy in male rats decreases the absolute refractory period measured in the stria terminalis, which connects the medial amygdala to the medial preoptic area (Kendrick and Drewett 1979, Kendrick 1982). The amygdala also projects to the ventromedial hypothalamus, but testosterone does not alter the electrophysiological characteristics of this projection in male rats, indicating a selective role of testosterone-mediated effects in the amygdala. It is interesting that all these neural regions are interconnected by well-

characterized pathways, such as the stria terminalis and medial forebrain bundle (see Figure 6), where both the neuroanatomical and neurophysiological evidence converge.

Effects of Various Androgens on Male Copulatory Behavior

The strongest evidence for the role of gonadal steroid hormones in mediating copulatory behavior has come from studies involving removal of the gonads followed by endocrine treatment (Beach 1948a). Systemic injections of testosterone gradually restore male copulatory behavior in gonadectomized male rats over the course of several weeks (Stone 1939). The roles of androgens in the medial preoptic area and amygdala have received considerable attention regarding the modulation of male copulatory behavior (Lisk 1967, Rezek and Whalen 1978, Nyby and Simon 1987, Kaplan and McGinnis 1989, McGinnis et al. 1989). Bilateral intracranial implants of testosterone propionate into the anterior hypothalamic region and medial preoptic area (Davidson 1966, Lisk 1967) can restore male copulatory behavior in gonadectomized rats. Furthermore, neural implants of testosterone only influence behavior and do not modify the hormone-dependent sex accessory organs, such as the prostate and seminal vesicles (Lisk 1967). This work is important because it firmly establishes the brain as the primary site of hormonally-mediated male copulatory behavior.

There has been a long-standing controversy over whether testosterone is the active agent in the brain, or if testosterone is just a precursor or prohormone (Perez Palacios et al. 1973). This is because testosterone can be converted to both dihydrotestosterone and estradiol in the rat brain (Celotti et al. 1991). When various androgens are given to gonadectomized male rats, only aromatizable androgens (such as testosterone) are able to stimulate male copulatory behavior (Beyer et al. 1973, Parrott 1975). Furthermore, either systemic injections or Silastic implants of very large amounts of dihydrotestosterone do not restore male copulatory behavior in gonadectomized rats (McDonald et al. 1970, Whalen

and Luttge 1971, Baum and Vreeburg 1973, Baum 1979), even though dihydrotestosterone is an androgen receptor agonist. A hypothesis for why treatment with dihydrotestosterone alone does not completely restore male copulatory behavior in rats is that it is rapidly metabolized to weak androgens (such as androstanediols) in the brain (Gay 1975).

Besides naturally occurring androgens (such as testosterone or dihydrotestosterone), several synthetic androgens have been tested for their ability to mediate male copulatory behavior. For instance, fluoxymesterone is a nonaromatizable synthetic androgen that does not restore male copulatory behavior in rats, even though it restores the condition of the seminal vesicles and prostate to intact levels (Beach and Westbrook 1968a). Methyltrienolone (R1881) is a synthetic steroidal androgen agonist which also does not undergo 5α-reduction, aromatization, or rapid metabolism in brain (Doering and Leyra 1984a, Doering and Leyra 1984b). R1881 binds and activates androgen receptors with a higher potency than testosterone (Dube et al. 1976, de-Boer et al. 1986, Van-Loon et al. 1988, Ojasoo et al. 1994). Since R1881 can activate androgen receptors in cultured cells of several types (and the androgen receptor is not believed to be different in the brain from peripheral androgen receptors), androgen-dependent behaviors should be stimulated by R1881. In spite of an early study indicating that R1881 did stimulate male copulatory behavior in gonadectomized rats, a more recent paper showed that this is not the case (Sodersten and Gustafsson 1980a, Baum et al. 1987). Lastly, the conversion of testosterone to dihydrotestosterone may not be necessary for male copulatory behavior because the synthetic androgen, 7α-methyl-19-nortestosterone (MENT, which cannot be 5α-reduced but can be aromatized to an active estrogenic steroid), is effective in restoring copulatory behavior in gonadectomized male rats (Morali et al. 1993). Therefore, these studies indicate that aromatized androgen metabolites, but not 5α-reduced metabolites, might play an important role in the restoration of male copulatory behavior in rats.

Effects of Various Estrogens on Male Copulatory Behavior

Interest in the effects of estradiol on male copulatory behavior probably originated in the work of Steinach (reviewed in 1940) and Moore (1919) on the reversal of copulatory behavior following transplantation of heterologous gonads (i.e., ovaries transplanted into males and testes transplanted into females). These studies indicated that adults of both sexes were sensitive to steroid hormones from the opposite sex. However, male and female rats occasionally exhibit aspects of copulatory behavior (i.e., mounting [male-typical] and lordosis [female-typical]) normally observed in the opposite sex (Stone 1923, Beach 1938a). The first studies in which estrogens were administered to gonadectomized male rats were conducted on animals that were prepuberally gonadectomized (Ball 1939, Beach 1942c). These studies showed that estrogen activated male copulatory behavior in prepuberal rats, while also increasing the level of female-like copulatory behavior in response to other intact males. The use of prepuberally gonadectomized rats offered the advantage of lower interanimal variability in response to endocrine therapy (Beach 1948a). The lower amount of interanimal variability in Beach's prepuberally gonadectomized rats was probably because the animals were hormonally naive (in the sense that the animals never experienced postpuberal brain androgen levels), along with the fact that the animals were sexually inexperienced.

Although systemic injections of estradiol have been found to stimulate male copulatory behavior in gonadectomized rats (Davidson 1966, Pfaff 1970, Feder et al. 1974), many studies testing the effects of estradiol, either alone or in combination with dihydrotestosterone, on male copulatory behavior have not consistently replicated this effect. For example, some studies indicate that estradiol is as effective as testosterone, while others indicate that estradiol is at best marginally effective in restoring male copulatory behavior to gonadectomized rats (Davidson 1969, Sodersten and Gustafsson 1980b, Kaplan and McGinnis 1989, McGinnis and Dreifuss 1989, Matochik and Barfield

1991, Rasia Filho et al. 1991). This variability could be attributed to the doses of estradiol administered (Kaplan and McGinnis 1989), because excessive estradiol doses potentially could result in nonselective and/or nonphysiological effects. Therefore, estradiol is not equivalent to testosterone in restoring male copulatory behavior in rats.

Since testosterone is metabolized to estradiol and dihydrotestosterone in the brain, and treatment with either metabolite alone does not restore male copulatory behavior, stimulation by both androgens and estrogens might be necessary for the restoration of male copulatory behavior in rats. Some studies have shown that when estradiol and dihydrotestosterone are given together, they can activate the full repertoire of male copulatory behavior (Feder et al. 1974, Baum et al. 1982, Clemens and Pomerantz 1982). When estradiol is implanted directly into the medial preoptic area-anterior hypothalamus in conjunction with systemic dihydrotestosterone injections, it can stimulate male copulatory behavior, but not when estradiol is implanted alone (Davis and Barfield 1979). However, implants of estradiol and dihydrotestosterone, in doses providing androgen and estrogen receptor occupation comparable to those found in intact males, have no effect on male copulatory behavior (McGinnis and Dreifuss 1989).

In theory, treatment with both estradiol and dihydrotestosterone should be equivalent to testosterone in restoring male copulatory behavior. However, it is possible that neurons containing aromatase could generate high intracellular concentrations of estradiol from the metabolism of testosterone, and that these intracellular estradiol levels would be higher than would be found in males treated with estradiol exogenously (i.e., without aromatization). Therefore, the amount of estrogenic stimulation in cells containing aromatase would not be the same in males treated with estradiol and dihydrotestosterone, as it would be in males treated with testosterone. This could be one reason why the "physiological" doses of estradiol and dihydrotestosterone, used by McGinnis and Dreifuss (1989), were not sufficient to restore male copulatory behavior in adult male rats.

If estradiol does not mediate the action of testosterone in the male rat brain, then what are the roles of androgens and estrogens in mediating male copulatory behavior? Beach proposed a mechanism for the neural control of male copulatory behavior in his seminal paper (Beach 1942a) on the factors of sexual excitement in male animals, called the central excitatory mechanism (c.e.m.). The idea was that gonadal steroid hormones (and also prior copulatory experience) lowered the threshold of the central excitatory mechanism for certain behaviors in the presence of an appropriate stimulus. For example, a sexually-receptive female would serve as the stimulus for an intact male rat to exhibit male copulatory behavior. Beach hypothesized that estrogen could lower the threshold of the central excitatory mechanism for male copulatory behavior in a manner similar to testosterone (Beach 1947b). However, Beach also recognized that estrogen was not equivalent to testosterone in stimulating the central excitatory mechanism, and the following quotation illustrates this point:

> The masculine response is possible because the c.e.m. has been rendered excitable by the estrogen, and because the threshold of the neural circuits mediating the masculine reactions is relatively low in the genetic male. However, this threshold is not as low as it would be under the influence of gonadal androgens, and consequently the masculine reactions of the estrogen-treated male castrate are sluggish and less intense than those of the intact male or the castrate treated with testosterone. (Beach 1942a p. 193)

Beach's central excitatory mechanism included a possible role for estrogen in mediating male copulatory behavior, but it did not provide a mechanism (Rose 1990) or establish that it was a physiological phenomenon. The evidence for a physiological role of estrogen in males came with the development of the aromatization hypothesis.

Origin of the Aromatization Hypothesis

The idea that estradiol could mediate male copulatory behavior did not actually come from studies on the hormonal activation of copulatory behavior in adult males. This idea came from studies about the organizing effects of steroid hormones on brain sexual differentiation. As stated earlier, testosterone produced in the testes organizes the brain in a

masculine fashion during early postnatal development in the rat. However, only aromatizable androgens or estrogens could masculinize the prenatal rat brain (Darrah et al. 1971, McDonald and Doughty 1974, Perez Palacios et al. 1975, Goy and McEwen 1980). Further work showed that estrogen antagonists (McDonald and Doughty 1972) and aromatase inhibitors (Houtsmuller et al. 1994) blocked testosterone's effect on brain masculinization. The "aromatization hypothesis" postulates that testosterone is a prohormone for the formation of estradiol in the brain (reviewed in Naftolin and MacLusky 1984). This hypothesis was strengthened by the discovery of aromatase activity in the brain (refer back page 10 for a discussion of the aromatase enzyme). The enzyme cytochrome P450 aromatase converts testosterone to estradiol and this enzyme is found in brain regions that are known to be important for male reproductive behavior. Furthermore, many of these brain regions also contain androgen and estrogen receptors.

The similarities between the hormonal activation of male rat copulatory behavior and the organizing effects of testosterone on the neonatal rat brain (i.e., the inability of nonaromatizable androgens to active male copulatory behavior or masculinize the brain) lead McDonald to propose that the conversion of androgens to estrogens was necessary for the activation of male copulatory behavior (McDonald et al. 1970, reviewed in Naftolin and MacLusky 1984). Thus, the aromatization hypothesis proposed that the neural effects traditionally attributed to testosterone (such as sexual differentiation of the brain and male copulatory behavior) were mediated by estradiol in the brain derived from testosterone in situ.

An additional piece of evidence supporting the role of aromatization in mediating male copulatory behavior came from studies with aromatase inhibitors. The first aromatase inhibitor that was developed was aminoglutethimide, and this compound blocked testosterone's restoration of male copulatory behavior in rats (Beyer et al. 1976b). Another aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), blocked male copulatory behavior in rats, and this effect was reversed by estradiol treatment (Christensen and

Clemens 1975, Morali et al. 1977). However, subsequent work has shown that these studies were confounded by ATD's nonselective inhibition of androgen receptors and the use of supraphysiological estradiol doses to counteract the effect of the aromatase inhibitor (Kaplan and McGinnis 1989). On the other hand, more recent studies with nonsteroidal aromatase inhibitors have addressed these problems, and indeed support the role of aromatization in mediating male rat copulatory behavior. The nonsteroidal aromatase inhibitor fadrozole is more selective than ATD with regard to blocking androgen receptors, and has a very low inhibitory constant (K_i ~ 1.6 nM, Bhatnagar et al. 1990, Vanden Bossche et al. 1994). Fadrozole has been shown to block preoptic aromatase activity in doves, monkeys, mice, and rats (Wozniak and Hutchison 1993). Fadrozole treatment for 2 weeks on testosterone-treated gonadectomized rats, in a maintenance paradigm, blocked male copulatory behavior by reducing ejaculations by 77% (Bonsall et al. 1992). Thus, studies with aromatase inhibitors have provided substantial support for the role aromatization in mediating male copulatory behavior.

Challenges To The Aromatization Hypothesis

Studies with androgen antagonists indicate that the role of androgen receptors in mediating copulatory behavior cannot be excluded. Androgen antagonist implant studies have shown that site-specific androgen receptor blockade impairs male copulatory behavior (McGinnis et al. 1996). However, early studies using implants of androgen antagonists into the medial preoptic area were confounded by the partial agonist activity and short half-life of the drugs (Liao et al. 1974, Earley and Leonard 1977, Freeman et al. 1989). In a manner similar to studies with aromatase inhibitors, more recently developed androgen antagonists overcame many of the problems associated with older androgen antagonists. Peripheral administration of hydroxyflutamide effectively inhibits cell nuclear androgen receptor binding and blocks the restoration of male copulatory behavior (McGinnis and

Mirth 1986). Blocking androgen receptors with intracranial implants of hydroxyflutamide in the medial preoptic area, but not in the amygdala or septum, also inhibits the restoration of male copulatory behavior (McGinnis et al. 1996). This indicates that the functional integrity of androgen receptors in the medial preoptic area is critical for the expression of male copulatory behavior. Moreover, blocking androgen receptors in the ventromedial hypothalamus virtually abolishes male copulatory behavior (McGinnis et al. 1996). This finding is of interest, because the ventromedial hypothalamus has been considered to be an important site for female copulatory behavior, but not male copulatory behavior in rats. The notion that the medial preoptic area is the site for male copulatory behavior is so ubiquitous that analysis of the ventromedial hypothalamus is often excluded in anatomical analyses (Lisciotto and Morrell 1993, Wood and Newman 1993a). The foregoing studies demonstrate regional specificity in that some, but not all, androgen concentrating brain areas contain androgen receptors that are necessary for the expression of male copulatory behavior.

Since the effects of testosterone and estradiol are traditionally believed to be mediated by their respective intracellular receptors (i.e., nuclear androgen and estrogen receptors), blocking estrogen receptors should block estradiol's effects on the restoration of male copulatory behavior. Efforts to block the effects of estrogens in the brain have resulted in mixed success. Studies with the nonsteroidal estrogen receptor antagonist MER-25 (ethamoxytriphetol) have not been effective in blocking male copulatory behavior (Baum and Vreeburg 1976, Beyer et al. 1976b). Mixed results have been reported for CI-628 (Luttge 1975, Yahr and Gerling 1978). A report that tamoxifen significantly depressed the restoration of male copulatory behavior is problematic because male copulatory behavior was not completely restored in the testosterone-treated controls (Beyer et al. 1976b). In addition, hydroxytamoxifen does not inhibit testosterone's restoration of male copulatory behavior in rats (McGinnis and Mirth 1986). This could either mean that estrogen receptor action is not involved in male copulatory behavior, or that these

antiestrogens were just ineffective in blocking brain estrogen receptors. Some shortcomings of the previous studies were that these antiestrogens are all partial agonists (Jordan 1984, Wakeling 1992), and that these compounds behaved like estradiol in nuclear exchange assays (so these assays were not helpful in determining whether the drugs were effective or not).

In conclusion, while the role for a testis-borne factor in mediating hormone-dependent sociosexual behaviors (especially male copulatory behavior) in adults has been known for some time, its exact nature is still not certain. Clearly, testosterone is getting to the brain and activating androgen receptors, but whether hormone-dependent sociosexual behaviors are mediated by testosterone and/or estradiol through their respective cell nuclear receptors is still an open question. Moreover, it is not known if similar mechanisms mediate the hormonal regulation of different hormone-dependent sociosexual behaviors in adult male rats.

Chapter 3 - General Methods

Subjects

Adult male (initial body weight 226-250 g) and female (initial body weight 175-200 g) Long Evans rats purchased from Charles River Laboratories (Wilmington, MA) were used in these studies. Long Evans rats, also known as hooded rats, are an outbred strain routinely used in reproductive behavior studies. These rats display high levels of copulatory behavior in the laboratory (McLean et al. 1972), are easily handled, and quickly recover from surgery.

The animals were individually housed in our laboratory's colony room at Mount Sinai's Center for Lab Animal Sciences (CLAS) in hanging stainless steel cages. Routine daily care for the animals was provided by CLAS personnel. The room's temperature was kept constant at 23° C, and the lights went on at noon and off at midnight (reverse 12:12 light:dark cycle) to make it easier to work with the animals when they were most active. Purina Laboratory Rodent Chow pellets and tap water were available ad libitum. Individual animals were identified by punching holes in the ears of anesthetized animals at specific locations during surgical procedures (McGinnis lab protocol). All experimental protocols were reviewed by the Mount Sinai Institutional Animal Care and Use Committee and conducted in accordance with the NIH guidelines for the care and use of laboratory animals (NIH Pub. No. 85-23. revised 1985).

Surgical Techniques

Anesthesia

For basic surgical procedures, including gonadectomy and implanting subcutaneous capsules, inhalational anesthetics were used. At the beginning of my work, the laboratory routinely used ethyl ether. However, due to ether's flammability, I was encouraged to use non-flammable inhalational anesthetics (Sebesteny 1971). Both Halothane (Halocarbon Laboratories, N. Augusta, SC) and Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) were used, but they were poor substitutes for ethyl ether. Halothane had a very short duration of anesthesia, coupled with its effective dose being too close to its lethal dose, and with Metofane, it was difficult to control the depth of anesthesia. Therefore, I resumed using ethyl ether for anesthesia, except that surgical procedures were performed in the lab's fume hood rather than at the bench. To anesthetize the rats, they were placed into a large glass desiccating jar with 5 ml of ethyl ether soaked into paper towels until the animal was unconscious. Anesthesia was maintained by placing the animal's nose near the mouth of a 100 ml beaker which had ethyl ether soaked cotton located at the bottom.

For intracranial implant surgery requiring a greater depth of anesthesia, a combination of ketamine (60 mg/kg, intraperitoneal injection [ip]) and xylazine (7.2 mg/kg, intramuscular injection [im]) was used (Wixson et al. 1987a, Wixson et al. 1987b). The drugs were purchased from Sigma Chemicals (St. Louis, MO) in crystalline hydrochloride form and dissolved in saline less than one week before use. These doses provided a period of surgical anesthesia for at least one hour with very low mortality. After weighing the animal, the ketamine injection was given first. Then after a few minutes, when the ketamine started to take effect (noticeable by the animal's swaggering gait), the xylazine injection was given. The animals were then ready for surgery approximately ten minutes later. If an

animal did not become unconscious during this period, it was allowed 15 more minutes for the anesthesia to take effect. If that extra time was insufficient, or if the animal showed signs of recovery before the surgery was completed, a single booster shot of only ketamine was given (0.1 ml of 100 mg ketamine HCl/ml IP). After the surgery was completed, the animals were placed on a warm heating pad until the animal regained consciousness to prevent hypothermia.

Gonadectomy

Males

All males in these studies were gonadectomized (GdX) at the beginning of the experiment so that serum androgen levels could be controlled as previously described (McGinnis et al. 1981, Krey and McGinnis 1990). Ethyl ether was used to anesthetize the rats. The surgical area was washed with 100% ethanol before midline scrotal incisions (0.5 cm) were made. The testes were then exposed using "rat tooth" forceps, tied off with 4-0 silk, and removed. The incision was closed with stainless steel wound clips.

Females

Stimulus female animals were ovariectomized (OvX) to prevent pregnancy and to allow for pharmacological manipulations of sexual receptivity (McGinnis et al. 1981). Ethyl ether was used to anesthetize the rats, and the surgical area was shaved and washed with 100% ethanol. The ovary on each side of the female was exposed using "rat tooth" forceps, after making bilateral retroperitoneal incisions (0.5 cm). The ovaries were then tied off with 4-0 silk, and removed. The incision was closed with stainless steel wound clips.

For the behavior tests, females with estradiol capsules (see the following section) were made sexually receptive with a subcutaneous (sc) injection of 500 μg progesterone/rat four hours before testing. Non-receptive females were ovariectomized and did not receive estradiol implants or progesterone injections.

Drug Delivery

Silastic Capsules

Steroid hormones were chronically administered with subcutaneous Silastic capsules (Dow Corning, Midland, MI) filled with crystalline steroids. Silastic capsules are a well established method for long-term steroid hormone administration (Stratton et al. 1973, Merkx 1984, Butera and Czaja 1989, Kaplan and McGinnis 1989). They provide a constant rate of drug infusion (zero order drug kinetics) for several weeks, without the stress of daily injections on the animal (Nash et al. 1978). In fact, testosterone delivered from Silastic capsules restores male copulatory behavior better than injections of testosterone (Sodersten et al. 1980).

The male rats used in these studies (except where noted) received two Silastic capsules (1.5 mm inner diameter x 10 mm long) filled with 100% crystalline testosterone. Testosterone-filled Silastic capsules prepared in this manner have been previously shown to deliver physiological levels of testosterone in adult male rats (Krey and McGinnis 1990). However, serum testosterone levels were measured in adult male rats with a Double Antibody ^{125}I Radioimmunoassay (RIA) Kit from ICN Biomedicals, Inc. (Costa Mesa, CA) in this work too. These results are presented in Table 6. Gonadectomized males had significantly lower (ANOVA (2,24) $F = 17.056$, $p < 0.0001$) serum testosterone levels than either intact males or gonadectomized males implanted with two, 10 mm testosterone-filled Silastic Capsules. However, serum testosterone levels in gonadectomized males

Table 6. Serum Testosterone Levels

	serum testosterone (mean ng/ml ± SEM)	*n*
gonadectomized adult males	0.070 ± 0.044 *	7
intact adult males	1.872 ± 0.342	6
gonadectomized adult males with two, 10 mm testosterone-filled Silastic Capsules	1.725 ± 0.196	14

* Fisher's Least Significant Difference Test, $p < 0.0001$

implanted with two, 10 mm testosterone-filled Silastic Capsules were not significantly different from intact males.

In some experiments, males were also implanted with one Silastic capsule (1.5 mm inner diameter x 5 mm long) filled with 1% crystalline 17β-estradiol in cholesterol to replace the estradiol that was lost in animals who did not have a substrate for aromatase (i.e., gonadectomized males who were not implanted with testosterone capsules) or in animals where aromatase activity was inhibited. These estradiol-filled capsules were prepared so that they would provide sufficient levels of estradiol to match the amount of brain cell nuclear estrogen receptor occupation found in intact male rats, as previously described in McGinnis and Dreifuss (1989) and also in Vagell and McGinnis (1997).

Stimulus female rats were implanted with one Silastic capsule (1.5 mm inner diameter x 5 mm long) filled with 100% crystalline 17β-estradiol one week prior to testing as previously described (McGinnis et al. 1981, Krey and McGinnis 1990). This estradiol-priming served to induce neural progestin receptors, that when stimulated with progesterone would facilitate female copulatory behavior (Etgen 1981, Vathy et al. 1987).

Intracerebroventricular Drug Infusion

For some of the experiments, steroid receptor or aromatase enzyme antagonists were administered directly into the right lateral cerebral ventricle (intracerebroventricular route [ICV]) by cannulas that were attached to miniature osmotic pumps (Alza Corporation, Palo Alto, CA; see Figure 7). The McGinnis lab has previously administered drugs directly

into specific brain regions (steroid receptor antagonists (McGinnis et al. 1996) and protein synthesis inhibitors (McGinnis and Kahn 1997)) by passive diffusion of drugs mixed with cocoa butter and bees wax from stainless steel cannulas. This method offers a way to deliver drugs into highly localized sites, but it is not well suited for delivering drugs into large areas or for tightly controlling the drug dose. Miniature osmotic pumps fitted to brain infusion cannulas get around these limitations by allowing the drug to be delivered into the cerebral ventricles, and they also make it easier to control for the drug dose, because the pumps deliver a constant rate of infusion. These pumps work by using osmotic pressure on a drug reservoir which causes them to pump 0.5 µl/hr of fluid from the reservoir for at least two weeks.

The cerebral ventricular system is the reservoir for the cerebrospinal fluid and is located beyond the blood-brain barrier. So if a drug is placed into the cerebrospinal fluid, it will be able to diffuse into the brain tissue. The reason the ICV method of drug administration was chosen was because one of the drugs, the antiestrogen ICI 182780, did not penetrate the blood-brain barrier. Therefore, in order to keep the drug delivery method constant among the different drugs used, all the antagonists in my initial studies were delivered by the ICV route (even though the other drugs such as fadrozole and hydroxyflutamide could penetrate the blood-brain barrier). There was one additional benefit to using ICV administration, which was to be able to separate the central nervous effects of steroid hormones from the peripheral effects of steroid hormones. For instance, with ICV administration of the antiandrogen hydroxyflutamide, I was able to separate androgenic effects that occurred in the brain, from those that were mediated peripherally in the male reproductive tract.

One common drug vehicle for ICV drug delivery has been synthetic cerebrospinal fluid (Sendelbeck and Urquhart 1985). However, several of the antagonists that I used (notably ICI 182780 and hydroxyflutamide) were extremely hydrophobic and would not dissolve in aqueous solutions. Therefore, it was necessary to deliver the drugs in an

organic solvent that was nontoxic and compatible with the interior of the miniature osmotic pumps. Propylene glycol was chosen at first because it has been previously used in the McGinnis lab to dissolve steroids for subcutaneous injections, and it has also been used for ICV delivery of steroids into the brain (Panek and Dixon 1985). However, hydroxyflutamide was only slightly soluble in propylene glycol, and after my pilot studies it became clear that a better solvent was necessary to deliver more hydroxyflutamide. The solvent that I finally chose was polyethylene glycol-200 (PEG-200), which had been previously demonstrated to be an excellent solvent for steroids (Will et al. 1980). Therefore, two different drug vehicles were used: propylene glycol in my first studies, and then PEG-200 in the rest. There was no detectable difference in the male copulatory behavior of animals receiving ICV infusion of propylene glycol or PEG-200 (data not shown). Therefore, I pooled the data from the ICV vehicle control animals that received propylene glycol or PEG-200.

The drug solutions were sterilized with 0.22 mm cellulose acetate syringe filters before the miniature osmotic pumps (Alzet model 2002, Alza Corporation, Palo Alto, CA) were filled, according to the manufacture's instructions. These pumps were then connected to brain infusion cannulas (28 gauge, Plastics One, Roanoke, VA) with 6 cm of Tygon tubing (TGY-020, Small Parts Inc., Miami Lakes, FL) and equilibrated in sterile saline for 24 hours at 37° C before implant surgery.

Intracranial Implant Surgery for Intracerebroventricular Drug Infusion

The brain infusion cannulas were fitted with the aid of a Kopf Small Animal Stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) following a protocol from Alza Corporation. The use of a specially designed electrode holder for the brain infusion cannulas (model #1776, David Kopf Instruments) facilitated the cannula placement. The implant site coordinates (see Figure 8) were 0.92 mm anterior to bregma, 1.6 mm lateral to

the midline, and 3.2 mm below the skull (atlas of Paxinos and Watson (1986)). The cannulas were secured with two stainless steel machine screws (size #0-80 x 3.2 mm) set in the skull, and glued with several layers of cyanoacrylate and dental cement. Each miniature osmotic pump was inserted into a subcutaneous pouch extending down the animal's back, and the incision was closed with stainless steel wound clips.

To control for my stereotactic surgery, a group of rats received methylene blue dye-filled miniature osmotic pumps to verify the cannula placement. In all cases examined, the cannulas were correctly positioned. However, I found that the dye diffused throughout the brain better with 22 gauge cannulas than 28 gauge cannulas. One possibility for why the dye diffused from the 22 gauge cannulas better than it did from the 28 gauge cannulas, was that the smaller cannulas could have been clogged more easily by cellular debris than the larger bore cannulas. In another control for cannula function, I injected 50 µl of methylene blue dye into the open end of the polyethylene tubing, connecting the cannula to the miniature osmotic pump, just after decapitation. Using this method, I was able to observe that a number of the cannulas had become blocked over the two week period they were in place. In animals where the cannula remained open, the dye distributed throughout the brain's ventricular system. However, each animal in the study did not get this control. Instead, I relied on biochemical assays (see page 82) to determine how effective the cannula placement and the inhibitors were, because whether or not the drug got to the tissue was secondary to whether the drug actually blocked what it was supposed to.

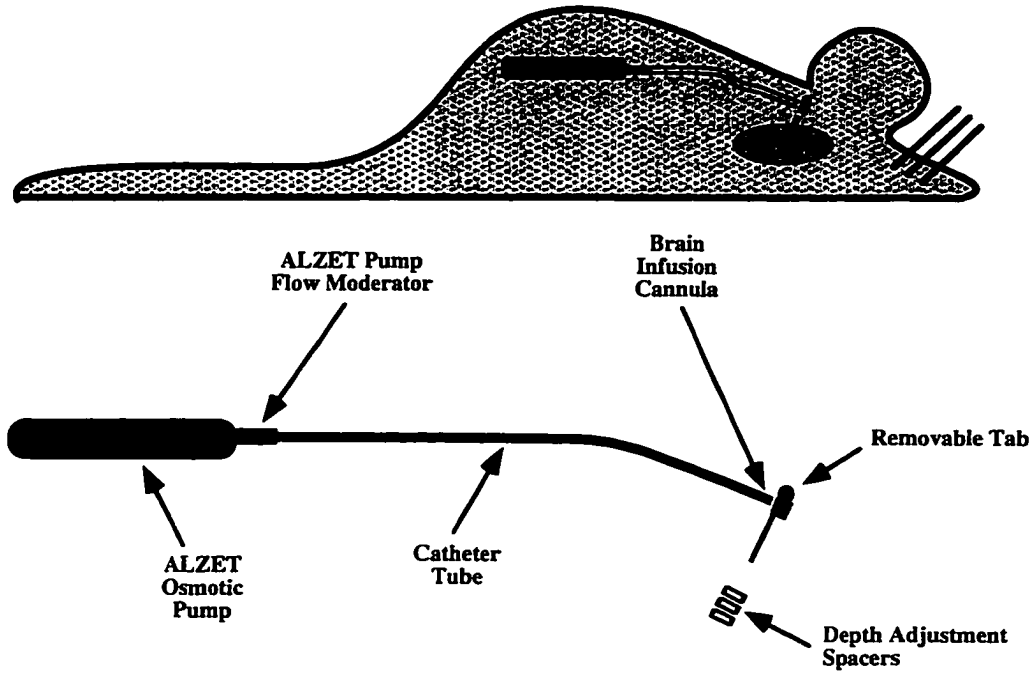


Figure 7. Miniature Osmotic Pump Diagram

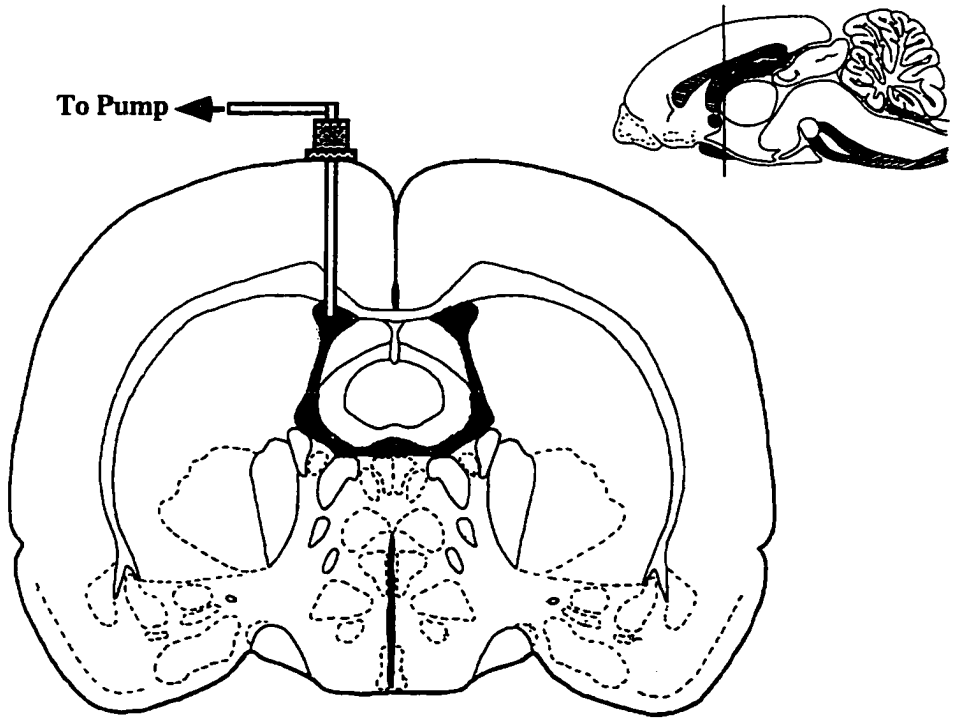


Figure 8. Intracerebroventricular Cannula Placement

Coronal section of a rat brain located 0.92 mm posterior to bregma. The lateral and third ventricles are shown in gray. The drawings were based on the following atlases: (Palkovits and Brownstein 1988, Paxinos and Watson 1996)

Chronic Subcutaneous Drug Delivery

In the last experiment, hydroxyflutamide (15 mg/day) was delivered with a miniature osmotic pump implanted subcutaneously on the animal's back. The hydroxyflutamide was dissolved in PEG-200, and Alzet model 2ML2 pumps were filled according to the manufactures instructions. This method allowed for a continuous infusion of hydroxyflutamide, which was used to avoid periods of low drug levels between injections. The abbreviation "scp" (*s*ub*c*utaneous *p*ump)was used to refer to this method of drug infusion, whereas the standard abbreviation of "sc" was used to refer to daily subcutaneous injections.

Behavior Tests

All behavior tests were conducted during the first eight hours of the animal's dark period under dim red incandescent lights in a separate room from where the animals were housed. This schedule of behavior testing was used because male rats exhibit a nocturnal rhythm in copulatory behavior (Harlan et al. 1980). Furthermore, since rodents are nocturnal animals, testing them during their subjective night (active period) is closer to their natural behavior pattern.

Behavioral Paradigm

There are generally two different behavioral paradigms that are used in studying the hormonal regulation of behavior. First, in a maintenance paradigm, experimental treatments are tested for their ability to maintain the level of male copulatory behavior when given immediately following gonadectomy. Second, in a restoration paradigm, the experimental treatments are tested for their ability to restore the level of male copulatory behavior that has been allowed to subside during a time period after gonadectomy. The overall difference between these two paradigms is that in a maintenance paradigm (see Figure 9A), the experiment begins while the animal is still under the influence of its endogenous hormones, while in a restoration paradigm (see Figure 9B), the experiment begins when the animal does not show any hormone-dependent behavior (as a result of the withdrawal of testosterone). Even though both of these approaches can be used to study similar aspects of hormone-dependent behaviors, they are not equivalent. For instance, systemic antiandrogen treatment has been shown to block testosterone's ability to restore male copulatory behavior, but the same dose of antiandrogen does not affect the maintenance of male copulatory behavior (McGinnis and Mirth 1986). Therefore, the underlying processes which mediate the maintenance and restoration of hormone-dependent behaviors might not

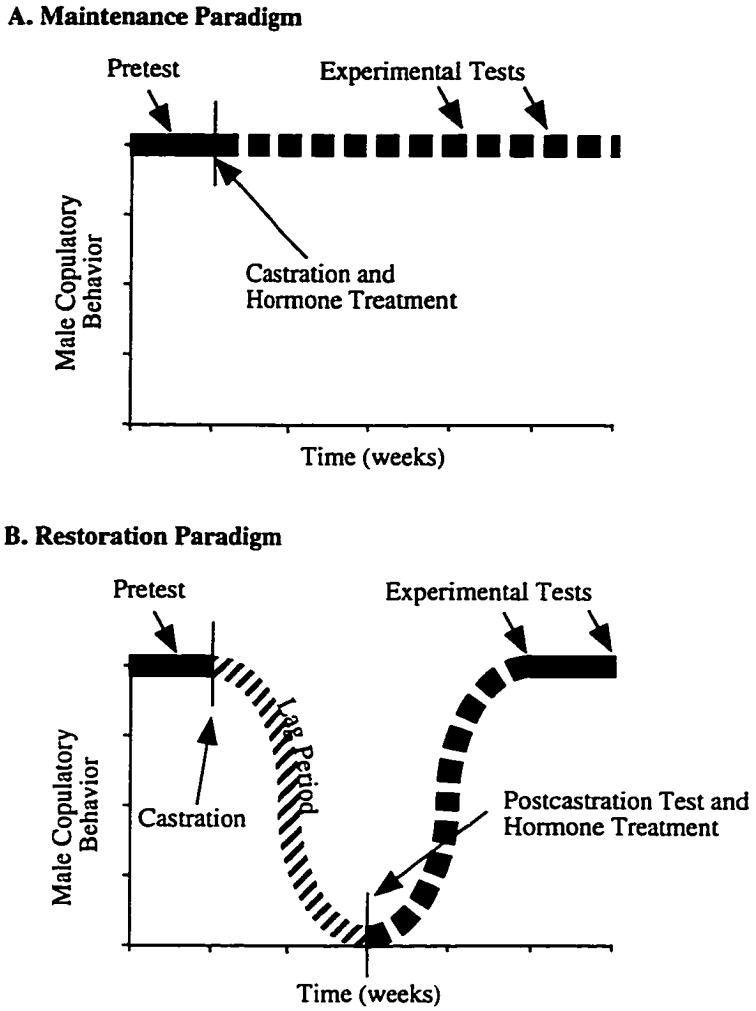


Figure 9. Diagrams of Maintenance and Restoration Paradigms

be the same. Since the restoration of hormone-dependent behaviors have been shown to be more sensitive to experimental manipulations than the maintenance of hormone-dependent behaviors, a restoration paradigm was chosen for this project.

Experimental Design

Upon receipt, the animals were pretested for male copulatory behavior to insure that they would mate in the laboratory. Normally, a few intact adult male rats from each shipment will not copulate even after repeated testing (Whalen et al. 1961). The criteria I

used for including individual animals in my work was that they complete at least one ejaculatory series (defined as the animal ejaculating within 30 minutes of mounting, followed by a postejaculatory interval of not more than 15 minutes). Animals which met these criteria were then gonadectomized (see surgical procedures) and allowed 2 weeks to recover. Actually, the animals recover from surgery within a few days, but a 2 week lag period following gonadectomy is necessary for the level of male copulatory behavior to decline (Stone 1927). After two weeks, the animals were retested for male copulatory behavior (postcastration test) to insure that they did not copulate (i.e., that the behavior was hormone-dependent). Our criteria for proceeding with the animals was that they not mount more than five times, and never intromit, during a 15 minute test. If the animals failed the postcastration test, they were retested a few days later. Animals meeting these criteria (before and after gonadectomy) were then placed into the study.

The experimental design of this project was to determine whether various receptor and enzyme antagonists would inhibit the ability of gonadal steroids to restore several hormone-dependent sociosexual behaviors, such as male copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking. In general, the animals were implanted with different combinations of steroid-filled Silastic capsules and received different enzyme/receptor antagonists for a 2 week duration. During that time, behavior tests were performed 7 and 13 days after implant surgery. The day 7 test served as an intermediate time point between the postcastration test (when the animals would not copulate, before any experimental treatments began) and the day 13 test (when the behavior would be completely restored in the control animals receiving testosterone alone).

In addition to male copulatory behavior, two other tests were performed, 50 kHz ultrasonic vocalizations/scent marking and partner preference, so that the hormone-dependence of these other behaviors could be compared to male copulatory behavior. All of these behavior tests were conducted on the same day. When conducting different behavioral tests on the same day, there is always the possibility that the animal's experience

in one test could have an influence on the animal's behavior in another test. Therefore, the order in which these tests were performed was kept constant throughout the project. The 50 kHz ultrasonic vocalizations/scent marking test was performed first, partner preference was performed second, and male copulatory behavior test was performed third. The idea was that in the 50 kHz ultrasonic vocalizations/scent marking test the male did not have any contact with other animals during the test, so it should have been performed first. Likewise, in the partner preference test the male was exposed to receptive and non-receptive females, but the male was prevented from intromitting with the receptive female during the test. So, the partner preference test was performed between the 50 kHz ultrasonic vocalizations/scent marking test and the male copulatory behavior test.

50 kHz Ultrasonic Vocalizations/Scent Marking

Ultrasonic vocalizations (50 kHz) and sebaceous scent marking were recorded at the same time in a 25 x 20 x 18 cm glass enclosure as described by Matochik and Barfield (1991). The enclosure was prepared by placing corn-cob bedding that had been soiled by several female rats (estrus bedding) into the chamber along with two, 50 ml glass jars to

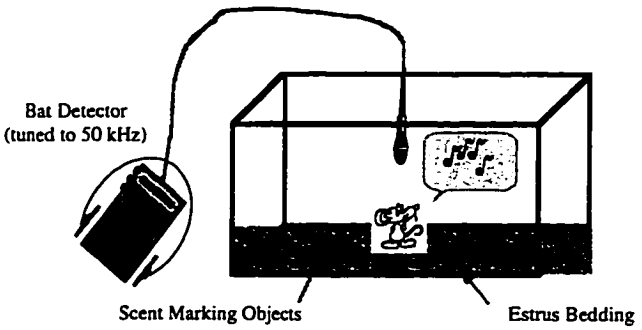


Figure 10. Vocalization/Scent Marking Apparatus

serve as scent marking objects. The estrus bedding was made-up on the day of testing by placing several progesterone-injected female rats (implanted with Silastic capsules filled with estradiol) into a Plexiglas cage with corn-cob bedding four hours before the test began. Ultrasonic vocalizations (50 kHz) were detected with a ultrasonic detector (QMC Model S100 Bat Detector) tuned to 50 kHz. These tests were conducted when the test subject was the only rat in the room. I shortened the test from ten minutes (Matochik and Barfield 1991) to five minutes because my preliminary work showed that there were no significant differences (data not shown) in the vocalization frequency during the first five minutes of the test as compared to the last five minutes of the test. During the test, the number of vocalizations (rapid chirps) and scent marks (defined as when the male dragged his perineum over the glass jars leaving a visible sebaceous mark) were recorded on a laboratory counter. An attempt was made at determining the duration of each vocalization manually with the use of a Macintosh computer as a timer. However, the duration of each vocalization was found to be under one second, and so it was not possible to manually determine the differences in individual vocalization duration.

Partner Preference

Partner preference tests were conducted in a three chambered clear Plexiglas apparatus (91 x 62 x 40 cm) consisting of a center chamber through which the animal had equal access to two end chambers (each chamber 29 x 62 cm). The outer chamber on one side contained a sexually receptive female while the other outer chamber contained a non-receptive female. Each female was tethered to her respective chamber by a Velcro or rubber band harness strapped to the female's abdomen (Williams et al. 1991). The vagina of the female was taped to prevent the males from intromitting during the test (Everitt 1990). At the start of the test, the male was placed in the center (empty) compartment and allowed

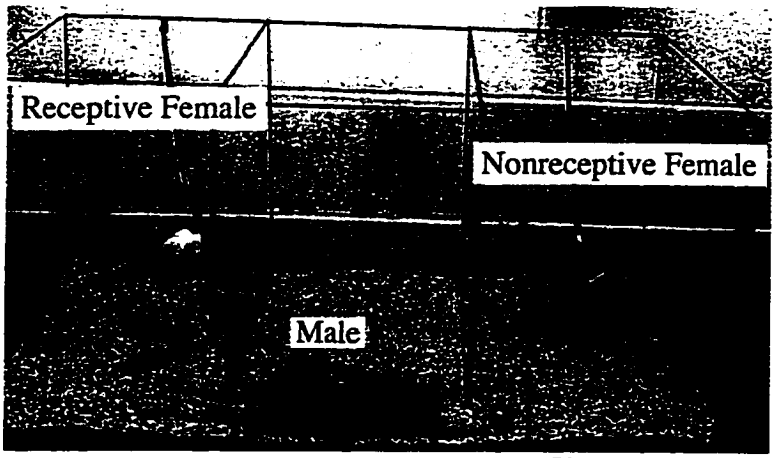


Photo by Ruby Rose

Figure 11. Partner Preference Test Apparatus

access to all chambers. The time the male spent in each chamber during the ten minute test was recorded by keying the animal's position into a computer (Vagell et al. 1995).

One aspect common to preference tests, regardless of the experimental paradigm, is the need to measure the time the animal spends in a given chamber. The most basic way to do this is with a stopwatch and just measure the time the animal spends in each chamber. On the other hand, elaborate automated setups to measure the amount of time the animal spends in each chamber have been presented in the literature (Bozarth 1983, Bakker et al. 1994). Both of these data collection methods have their limitations. The manual method is tedious especially for experiments involving more than two chambers, and automated methods often require costly and dedicated equipment. I developed a computer program (Vagell et al. 1995) to facilitate data collection in place preference tests on a Macintosh computer where the investigator keys in which chamber the animal is located in (the program's manual is located in the Appendix B). The program offers simplicity in that it does not require any dedicated equipment and does not involve any electronic components to configure. The program's advantages are that it facilitates data collection so that multiple tests can be performed at the same time, it simplifies data collection, and determines the

number of times the animal crosses a chamber boundary to monitor the animal's activity level.

Male Copulatory Behavior

Male copulatory behavior testing was conducted in 25 x 20 x 18 cm glass enclosures following a previously established protocol (McGinnis and Gorski 1980, McGinnis and Mirth 1986, McGinnis et al. 1989). Mounting was scored when the male made pelvic thrusts on top of the female. Intromissions were defined as mounts with penile insertion. Male rats have a stereotypical behavior pattern which allows for mounts to be differentiated from intromissions (Young 1961). When a male mounts a female, he often either moves away slowly or stays in place while the female scrambles out from under him. Following an intromission, the male falls back onto his hindquarters. Therefore, one can differentiate between a mount and an intromission. After mounting or intromitting, the male grooms himself. This stereotypical behavior pattern is depicted in Figure 12.

The behavioral criteria used to score ejaculations is similar to scoring an intromission, except that during an ejaculation the male spends more time (1-3 seconds) crouched over the female before he dismounts. Following an ejaculation, the male sits by himself for a period of about 4-7 minutes and vocalizes with long song-like calls at 22 kHz (Barfield and Geyer 1975). During this refractory period (the postejaculatory interval) the male will not reinitiate copulation. The end of the postejaculatory interval is defined as when the male either mounts or intromits the female again. This whole process from the first mount until the end of the postejaculatory interval is defined as one ejaculatory series.

Standard measures of male copulatory behavior (see Table 7) were obtained during the tests (Sachs and Barfield 1970, Sachs and Barfield 1974, McGinnis and Mirth 1986, Kaplan and McGinnis 1989, McGinnis et al. 1989). All males were placed into the test arena 5 minutes before the female for them to get accustomed to the test environment. Latencies (mounts, intromission, and ejaculation) were recorded from the time the female was placed into the arena (this marked the beginning of the test). It should be noted that

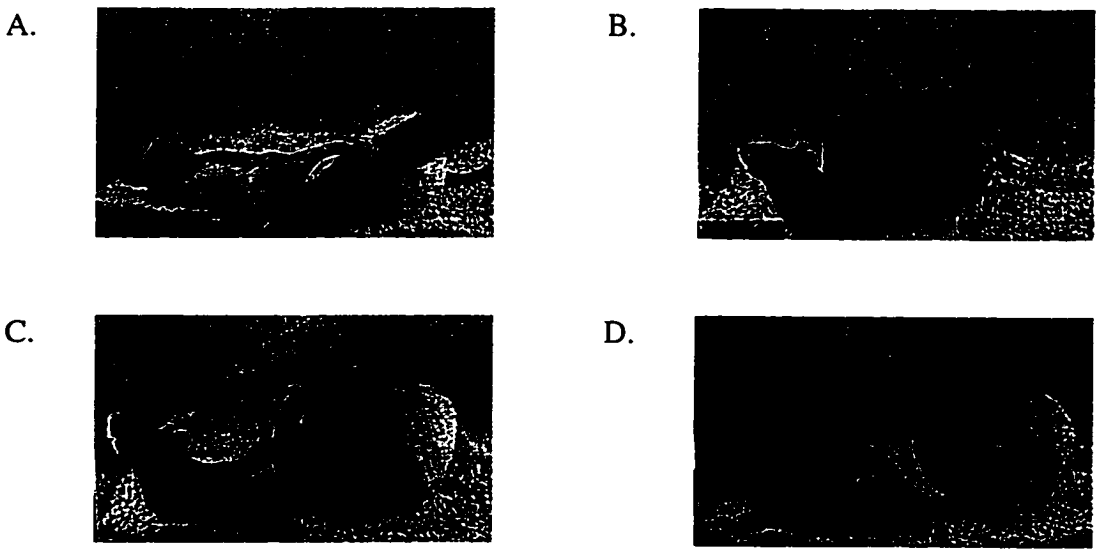


Figure 12. Pictorial Sequence of Copulatory Behavior in Rats

Panel A shows the male (on the right side) investigating a sexually receptive female (on the left side) who is oriented away from the male. Panel B shows the male just as he begins to mount, and then in panel C dismount without intromitting. Panel C nicely illustrates the lordosis posture (the curved arching of the back) of the female. Panel D shows the male grooming himself. Although it is not shown in the still photograph in panel D, the female rat's ears would be vibrating rapidly and she is crouched and ready to dart away (these are two examples of proceptive behaviors by the female).

Table 7. Male Copulatory Behavior Measures

Parameter	Abbr.	Definition
Mount Frequency	MF	number of mounts
Mount Latency	ML	duration from the beginning of the test until the first mount
Intromission Frequency	IF	number of intromissions
Intromission Latency	IL	duration from the beginning of the test until first intromission
Ejaculation Latency	EL	duration from the beginning of the test until ejaculation
Postejaculatory Interval	PEI	duration from ejaculation until first mount or intromission
Hit Rate	HR	$\left(\frac{\text{intromssion frequency}}{\text{mount frequency + intromssion frequency}}\right) \times 100$
Intercopulatory Interval	ICI	$\frac{\text{ejaculation latency - intromission latency}}{\text{intromission frequency}}$
Copulatory Duration	CD	ejaculation latency - intromission latency

ejaculation latency is often defined as the time from the first intromission until ejaculation (Sachs 1978, Pfaus et al. 1990), but in the McGinnis lab, ejaculation latency is defined as the time from when the female is introduced, at the beginning of the test, until ejaculation (McGinnis and Kahn 1997, Vagell and McGinnis 1997). However, the parameter labeled "copulatory duration" is analogous to the ejaculation latency in some reports.

The postejaculatory interval (PEI) was defined as the time duration from ejaculation to either the next mount or intromission. From the raw behavioral data, other derived measures such as hit rate (HR), intercopulatory interval (ICI), and copulatory duration (CD) were determined to assess the animals' copulatory efficiency and pace (see Table 7). Stimulus females were rotated every 5 minutes to randomize the female's influence on the test. Tests were terminated when either intromission latency, ejaculation latency, or PEI exceeded 15 min.

Biochemical Assays

Cell Nuclear Receptor Exchange Assays

Nuclear exchange assays are used to measure the amount of ligand-bound cell nuclear steroid receptors, by exchanging the endogenous bound ligand for an exogenous radiolabeled-ligand in vitro (Roy and McEwen 1977, McGinnis et al. 1983). This kind of assay is useful because it provides a measure of receptor functionality in vivo, by determining the amount of "activated" or "transformed" receptors in the nucleus. Studies involving nuclear exchange assays have shown that gonadectomy decreases nuclear androgen receptor levels, and that testosterone treatment restores nuclear androgen receptors to precastration levels (McGinnis et al. 1983, 1986, Handa et al. 1988, Kaplan and McGinnis 1989, Roselli 1991, 1992). This is reasonable, since in the absence of testosterone, ligand-activated androgen receptors would not be expected to be found in the nucleus. Moreover, gonadectomy also results in an increase of limbic, pituitary, and seminal vesicle cytosolic androgen binding (McGinnis et al. 1983).

Nuclear exchange assays were used to quantitate the level of cell nuclear androgen (McGinnis et al. 1983) and estrogen (McGinnis et al. 1981) receptor occupation using previously established protocols in the McGinnis laboratory. Briefly, tissue homogenates were prepared from gross brain dissections (unless otherwise noted: pooled amygdala, hypothalamus, preoptic area, and septum; see Figure 13) and pituitary tissue with a glass-Teflon homogenizer at 0° C. Cell nuclei were purified with differential centrifugation through sucrose step gradients and then salt extracted to isolate the "transformed" receptors. The salt extract containing the receptor complexes was then incubated with either 4 nM ^{3}H-R1881 (Bonne and Raynaud 1975) overnight at 4° C with and without a 100 fold excess of dihydrotestosterone to correct for non-specific binding (androgen receptor

assays), or 2 nM ^{3}H-estradiol for 5 hours at 24° C and then overnight at 4° C with and without a 100 fold excess of diethylstilbestrol (DES) to correct for non-specific binding (estrogen receptor assays). Bound radiolabeled-steroid was separated from free radiolabeled-steroid by Sephadex LH-20 chromatography and then measured with scintillation counting. The results were corrected for cell number by normalizing the fmol radiolabeled-steroid bound to mg cellular DNA (Burton 1956).

In addition to the nuclear exchange assays, seminal vesicle weights were used as a bioassay for peripheral androgen action. For example, treatment of intact males with antiandrogens, or removing the gonads, resulted in a regression of seminal vesicle tissue (Whalen and Edwards 1969). The seminal vesicles were removed at the time of the steroid receptor assays, pressed to remove the seminal fluid, and then weighed. These organ weights are often presented with a correction for the animal's body weight (i.e., grams seminal vesicle per 100 grams body weight). However, there was no correlation (p = 0.1917) between the animals' body weights and the weights of their seminal vesicles, so unmodified wet tissue weights were reported.

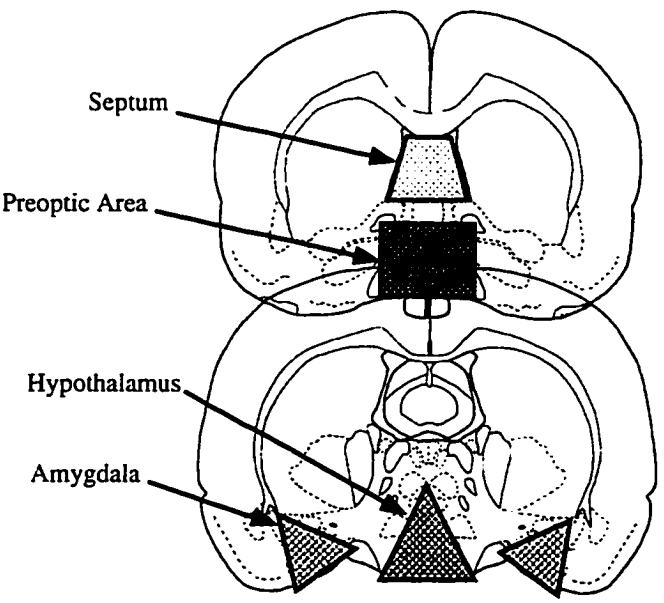


Figure 13. Limbic Brain Regions Pooled for Nuclear Exchange Assays

Data Analysis & Statistical Methods

The experimental data were analyzed using the following Macintosh software (Apple Computer, Inc., Cupertino, CA): Excel for the Macintosh v5.0 (Microsoft Corporation, Redmond, WA), StatView v4.5 and SuperANOVA v1.11 (Abacus Concepts, Berkeley, CA), and SPSS v6.1.1S (SPSS Inc., Chicago, IL). The overall Type I error level (α) was set at 0.05 (i.e., the chance of falsely rejecting the null hypothesis). Kolmogorov-Smirnov Normality tests were used to test the hypothesis that the data fit normal distributions. Levene's test and Bartlett-Box's F test were used to test for homogeneity of variance, and Box's M test was used to check for homogeneity of the dispersion matrices in the repeated measures tests. However, the results for these tests were not reported. Group means and the standard error of mean (SEM) are shown in the figures displaying normally distributed data.

Discussion of data analysis for the 50 kHz vocalization and scent marking tests is covered in a separate section (page 88), because the analysis of these data required special procedures. For the rest of the data, standard statistical tests were used, such as ANOVA and t tests, as defined in the manuals for SuperANOVA, StatView, and SPSS. Chi-square (χ^2) tests were used to test the hypothesis of independence for the percentage of animals mounting, intromiting, and ejaculating. Post hoc cell contributions (Everitt 1977) were then used to determine which of the observed group values significantly differed from the expected values in multiway contingency tables. This provided a way to find out which groups contributed to the overall Chi-square probability value when more than two groups were compared. Specific comparisons between the experimental groups and the control group (testosterone-treated gonadectomized males) were made with Fisher's Exact Probability tests.

Post hoc analyses were performed with Fisher's Protected Least Significant Difference (PLSD) test for pairwise comparisons and Dunnett's test for comparing the

experimental groups to a single control group. It should be noted that Fisher's PLSD test does not control the overall type I error level as well as more conservative tests such as Scheffé's test or some Bonferroni-type procedures (Keselman et al. 1991). However, there are two reasons for why Fisher's PLSD test was used in this work: First, the comparisons were established before the data were analyzed, so that multiple tests were not used indiscriminately (Saville 1990). Second, many post hoc procedures that guard against inflating the overall type I level of error do so at the expense of statistical power. Even though the type I level of error is controlled for in many of these tests, the type II level of error is increased (i.e., the chance of falsely accepting the null hypothesis). Since small group sizes were generally used in these experiments, Fisher's PLSD test was used with the type I level of error set at 0.05 to ensure that there was adequate statistical power for the analyses.

Partner preference data was generated by subtracting the time the male spent in the chamber with the non-receptive female from the time spent in the chamber with the receptive female (Edwards and Pfeifle 1983). Positive values indicate that more time was spent in the chamber with the receptive female while negative values indicate that the male spent more time with the non-receptive female. The partner preference scores (time with receptive female - time with nonreceptive female) were analyzed by repeated measures ANOVA. The values reported for the F tests are based on univariate analyses without corrections for correlated observations, because the F test values modified with Greenhouse-Geisser's epsilon statistic were not different from the uncorrected values. Planned comparisons for Group x Test interactions were made by testing the hypothesis that the means of the between-group factors were different, depending on the level of the within-group factors. These comparisons were made between each experimental group and the testosterone-treated group with contrast tests. In addition, Least Squares Mean Tests (which are similar to t tests) were used to test whether the values on test #1 and test #2 significantly differed from those on the postcastration test within each group. For example,

gonadectomized animals treated with testosterone had significantly greater partner preference scores on the tests 7 and 13 days after implant surgery. These significant differences are reported in the partner preference figures by asterisks above the columns.

All behavior testing was performed "blind" (i.e., the animal's number was noted only after the test was completed to avoid experimenter bias). For the individual measures of male copulatory behavior (refer back to Table 7), only data for animals who copulated were analyzed. So if an animal did not mount, there would be no mount frequency or mount latency to analyze. In addition, if an animal did not mount, the mount frequency cell in the table would be left blank, rather than having a mount frequency value of zero. This was performed in this way so that in groups with few males copulating, the group's mount frequency mean value would not be skewed by the zero values from the animals who did not copulate. Moreover, this definition concerning data collection is important because a low number of mounts in males who exhibited low levels of copulatory behavior would not be interpreted to mean the same thing as in males who ejaculated, but with a low number of mounts.

The animals were randomly selected into treatment groups. However, it should be noted that the animals used in this project were not from a random sample, but from a population that was preselected in the laboratory for copulatory behavior based on the criteria described on page 74. The criteria of hormone-dependent male copulatory behavior was used in preselecting animals for the partner preference, scent marking, and 50 kHz ultrasonic vocalization tests, because these same animals were also used for the study of male copulatory behavior. No additional selection criteria were used for the partner preference, scent marking, or 50 kHz ultrasonic vocalization tests.

The order of tests that were performed on the same day was kept constant throughout the experiment (as mentioned on page 76). In addition, the data that were collected when different tests were conducted on the same day (i.e., when male copulatory behavior was the only test performed vs. when 50 kHz ultrasonic vocalizations/scent

marking, partner preference, and male copulatory behavior were all measured) were only compared to data that was collected when the same tests were performed that day. For example, during my first experiments I only measured male copulatory behavior, so this data was not compared with data that was collected in my latter experiments when I measured 50 kHz ultrasonic vocalizations/scent marking, partner preference, and male copulatory behavior all on the same day. The reason the order of testing and type of tests that were performed in each session was important, was because putting the male in a sexual context, such as in the 50 kHz ultrasonic vocalization/scent marking and partner preference tests, facilitated male copulatory behavior. Figure 14 shows the effect of a prior 50 kHz ultrasonic vocalization/scent marking test and/or a partner preference test on copulatory duration (the time from the animal's first intromission to ejaculating; ANOVA, F (2,24) = 4.194, $p < 0.0274$). This trend was also evident for mount and intromission frequency and ejaculation latency, but these measures were not statistically significant.

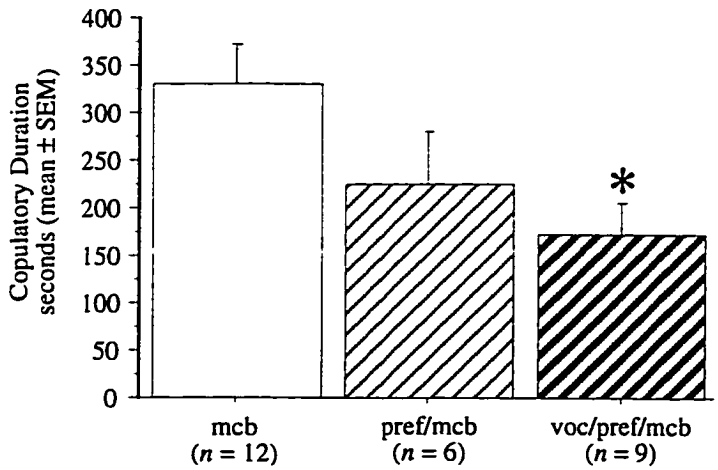


Figure 14. Effect of Partner Preference and 50 kHz Vocalization/Scent Marking Tests on Male Copulatory Behavior

mcb = male copulatory behavior test performed by itself
pref/mcb = male copulatory behavior test performed after a partner preference test
voc/pref/mcb = male copulatory behavior test performed after a 50 kHz ultrasonic vocalization/scent marking test and a partner preference test
* $p < 0.05$ vs. mcb, Fisher's PLSD post hoc test

Analysis of 50 kHz Vocalization and Scent Marking Data

The only data that needed special attention (with respect to violating test assumptions) were for the 50 kHz vocalization and scent marking tests. This was because these data were not normally distributed (Kolmogorov-Smirnov Normality tests, $p < 0.05$). Histograms representing 50 kHz vocalization and scent marking data for the precastration and postcastration tests are displayed in Figure 15 and Figure 16, respectively. The lines across the figures display comparisons of normal curves, based on the sample means and standard deviations. These figures show how the data are positively skewed. Mathematical transformations ($\log_{10}$ (x + 1) or $\sqrt{x}$) did not improve fitting the data to a normal distribution or solve the problem of heterogeneous variance (the between-group variance was significantly different, Levene's Test, $p < 0.05$). Therefore, standard tests based on normal distributions, such as ANOVA, were not appropriate for these data, and nonparametric tests were used on the raw data.

The hormone-dependence of both 50 kHz vocalizations and scent marking have been shown previously (Matochik and Barfield 1991). However, since this was the first

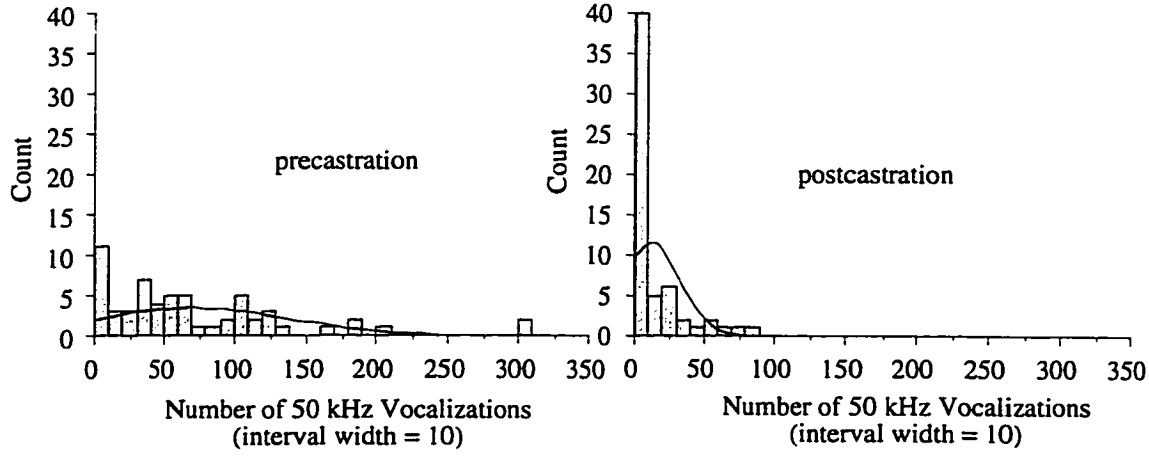


Figure 15. Histograms of 50 kHz Vocalizations Before and After Gonadectomy with Normal Curve Comparisons

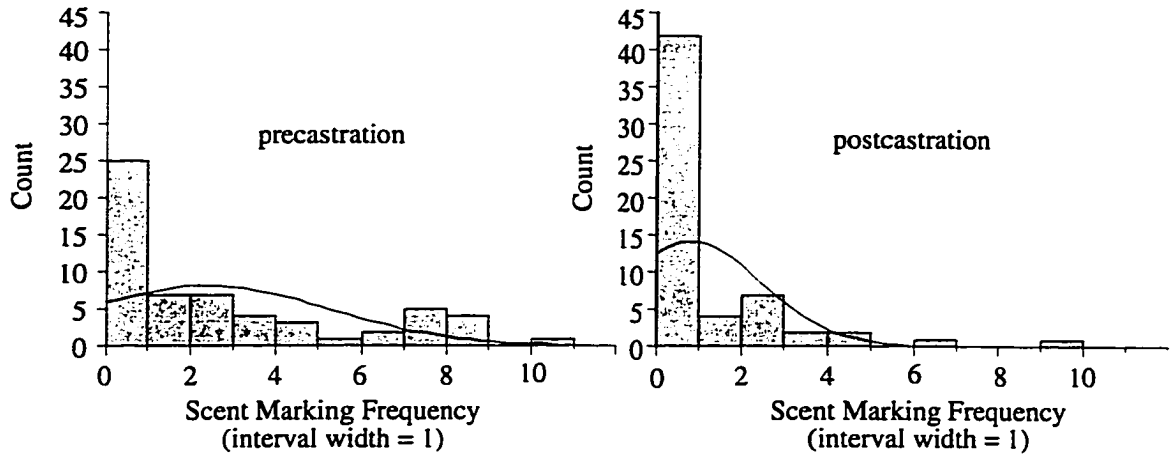


Figure 16. Histograms of Scent Marking Before and After Gonadectomy with Normal Curve Comparisons

time these measurements were made in the McGinnis Lab, the hormone-dependence of these behaviors was verified. The box plots in Figure 17 show that gonadectomy results in a significant decrease in the amount of both 50 kHz vocalizations (Wilcoxon Signed Rank Test, $N = 59$, tied Z-value = -5.79, $p < 0.0001$) and scent marking (Wilcoxon Signed Rank Test, $N = 59$, tied Z-value = -5.79, $p < 0.0001$). Box plots, which show percentile scores rather than the mean ± standard error of measurement, were used to display these data because the sample means were not good measures of central tendency for these datasets (as evident in the nonsymmetrical distributions in Figure 15 and Figure 16).

While my data are in agreement with Matochik and Barfield (1991) that gonadectomy results in a suppression of 50 kHz vocalizations and scent marking, I did not find that logarithmic transformations of the data were appropriate to allow for using parametric statistics based on a normal distribution. As a result, distribution-free tests, such as the Wilcoxon Signed Rank test, were used for the data in Figure 17, rather than parametric tests, such as a paired t test. In addition, a subsequent study by Matochik and Barfield (1994) indicated that nearly half animals tested after gonadectomy did not show a gonadectomy-dependent decrease in 50 kHz vocalizations and scent marking. However,

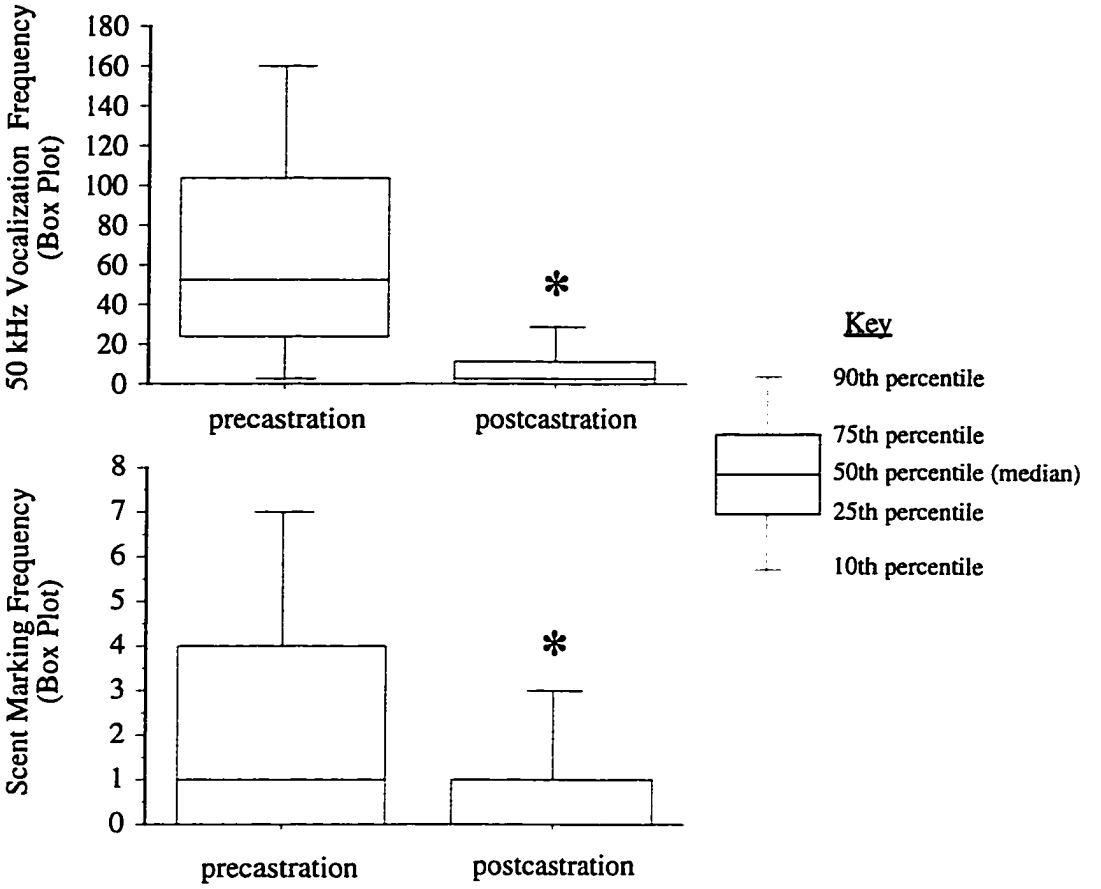


Figure 17. Box Plots of 50 kHz Vocalization Frequency and Scent Marking Before and After Gonadectomy

* $p < 0.0001$, Wilcoxon Signed Rank test

only 7 out of 59 animals vocalized more after gonadectomy, and only 8 out of 59 animals scent marked more after gonadectomy. Since my animals did not display this kind of stratification, no additional criteria were used in selecting animals, other than the ones defined for the study of male copulatory behavior (page 74).

One striking feature of the 50 kHz vocalization and scent marking data is how much each animal differed from one another, even before the experiment began (see Figure 15 and Figure 16). Ordinarily, interanimal variability is not so much of a problem because it

can be accounted for using a repeated measures ANOVA. However, the data for the 50 kHz vocalization and scent marking tests violated all three assumptions for repeated measures ANOVA (i.e., normality, homogeneity of variance, and homogeneity of covariance). Even though ANOVA is generally robust to violations of these assumptions (Stevens 1996), a repeated measures ANOVA on the raw data was not appropriate, due to the degree that these assumptions were violated. In the analysis for Figure 17, this problem was overcome by using the Wilcoxon Signed Rank test, which is a distribution-free test on the paired differences for each set of observations. Unfortunately, for mixed model designs with between- and within-subject comparisons, few nonparametric tests are available and these are subject to many additional underlying assumptions. Therefore, an alternative approach was used by analyzing the paired difference scores for each set of observations.

In spite of that fact that the raw data deviated from normality, transformation of the data to paired difference scores reduced the heterogeneity of variance and corrected the normality problem. This allowed for between-group comparisons to be made, while still taking into account that the data were from replicate samples, with parametric statistics. For example, each observation in a column was subtracted from its previous observation on the same row (see Table 8). This resulted in two new sets of paired difference scores. Standard one-way ANOVAs were then used to test the hypothesis that the groups means of the paired difference scores were different from each other on both sets of difference scores. Therefore, the response of each animal across two tests was taken into account in the analysis. Partitioning the experiment into two components (test #1 - postcastration and test #2 - test #1) also provided a way to examine how each treatment differentially affected testosterone's restoration of 50 kHz vocalizations and scent marking, without making any assumptions about the patterns of the responses. For example, the data shown Table 8 represent the restoration of 50 kHz vocalizations in gonadectomized males treated with testosterone. One-sample t tests indicated that both sets of difference scores significantly deviated from zero ($p = 0.0015$ and $p = 0.0118$, respectively). This indicates that

Table 8. Example of How the Paired Difference Scores Were Calculated

Raw Data			Paired Difference Scores	
postcastration	test #1	test #2	test #1 - postcastration	test #2 - test #1
1	77	158	76	81
0	0	0	0	0
5	111	121	106	10
1	102	247	101	145
2	32	181	30	149
0	110	171	110	61
0	115	238	115	123
1	14	11	13	-3
1	124	155	123	31
		mean difference ± SEM	75 ± 16	66 ± 20

gonadectomized males treated with testosterone continue to vocalize more on each subsequent week of treatment. Consequently, this also indicates that the restoration of 50 kHz vocalization behavior is not complete after one week of testosterone treatment. However, this is not the case for scent marking, because the mean difference in scent marking for the interval of the first week was significantly different from zero (mean difference = 6, $p = 0.0006$), while the interval for the second week was not significantly different from zero (mean difference = 1, $p = 0.7500$). Therefore, the restoration of scent marking was complete after one week of testosterone treatment.

Chapter 4 - The Role of Aromatization in the Restoration of Male Rat Copulatory Behavior and Partner Preference

Introduction

The aromatization hypothesis of male copulatory behavior postulates that testosterone acts in the brain by aromatization to estradiol (Naftolin and MacLusky 1984). Testosterone is converted to estradiol by cytochrome P450 aromatase in many brain regions known for their role in mediating male copulatory behavior, such as the preoptic area and the amygdala (Simpson et al. 1994, Lephart 1996). However, estradiol's role in the expression of male copulatory behavior has been a controversial subject, because many studies testing the effects of estradiol on male copulatory behavior have not been consistent (Harding 1986). For example, in some studies (Davidson 1969, Sodersten 1973, Christensen and Clemens 1974) estradiol was found to be as effective as testosterone, while in others (Baum and Vreeburg 1973, Larsson et al. 1973b, Larsson et al. 1973a, Larsson et al. 1976, McGinnis and Dreifuss 1989, Matochik and Barfield 1991) estradiol was only marginally effective in restoring male copulatory behavior to gonadectomized rats. Significant factors contributing to this controversy have been the wide variety of estradiol doses used and the different methods of hormone administration (such as injections, Silastic capsules, or intracranial implants). This has made comparisons between these studies on the efficacy of estradiol in mediating male copulatory behavior difficult.

On the other hand, nonaromatizable androgens, such as dihydrotestosterone, are not sufficient for the restoration of male copulatory behavior (Baum and Vreeburg 1973, Beyer et al. 1973). Moreover, there is a possibility that high doses of dihydrotestosterone may be able to restore male copulatory behavior because one metabolite of

dihydrotestosterone, 3β-diol, is estrogenic (Morali et al. 1994). Only one study (Sodersten and Gustafsson 1980a) reported that methyltrienolone (R1881), a nonaromatizable androgen, is as potent as testosterone, but two others contradict this work (Baum 1979, Baum et al. 1987). In addition, the synthetic androgen, 7α-methyl-19-nortestosterone (MENT), which is not 5α-reduced but still can be aromatized, effectively restores male copulatory behavior (Morali et al. 1993). Since several studies have shown that male copulatory behavior is not affected by 5α-reductase inhibitors, 5α-reduction may not be necessary for male copulatory behavior in gonadally intact animals (Bradshaw et al. 1981, Sodersten et al. 1986, Wise et al. 1991). In spite of that fact that neither estrogens nor nonaromatizable androgens administered alone are very effective in restoring male copulatory behavior, low doses of estradiol combined with dihydrotestosterone are just as effective as testosterone in rats (Baum and Vreeburg 1973, Larsson et al. 1973b, Larsson et al. 1973a, Feder et al. 1974, Larsson et al. 1976, Sodersten et al. 1988, Morali et al. 1994). This led to the idea that male rat copulatory behavior could be mediated by the combined action of both testosterone and estradiol (Perez Palacios et al. 1975, Beyer et al. 1976a).

The goals for this study were to determine if selectively blocking aromatization would inhibit the ability of testosterone to restore male copulatory behavior, and if this effect could be reversed by simultaneous treatment with a physiological dose of estradiol. I used the aromatase inhibitor, fadrozole (Steele et al. 1987, Vanden Bossche et al. 1994), administered into the right lateral ventricle of gonadectomized adult male rats to test if blocking aromatase would inhibit testosterone's restoration of male copulatory behavior and partner preference. Fadrozole has been previously shown to reduce male rat copulatory behavior in a maintenance paradigm, and it does not block androgen receptors (Bonsall et al. 1992, Clancy et al. 1995). Cell nuclear estrogen receptor exchange assays were used to assess the effects of fadrozole on nuclear estrogen receptor occupation, and to insure that the estradiol dose was within the normal range of estrogen receptor occupation found in

gonadally intact male rats. Fadrozole's effects on nuclear androgen receptor occupation were also measured to confirm that fadrozole did not block brain androgen receptors.

Experimental Design

Prior to the experiment, all males were pretested for male copulatory behavior as described in the General Methods Chapter (page 79). The first criteria for including males in the study was that they complete at least one ejaculatory series. The males were then gonadectomized under ethyl ether anesthesia, and retested two weeks later for male copulatory behavior (postcastration test). The second criteria for inclusion into the study was that the males mount less than five times, without intromitting, in the postcastration behavior test. Animals meeting these two criteria were then implanted with miniature osmotic pumps fitted to brain infusion cannulas, and Silastic capsules filled with combinations of testosterone and/or estradiol.

This work was performed in two parts. The first was a pilot study to determine the optimal ICV dose of fadrozole, because this method of drug delivery had not been attempted before with fadrozole. Since this was a pilot study, data from only one male copulatory test was collected after 13 days of treatment. Once the optimal dose was determined, the experiment began. The animals were tested for partner preference and male copulatory behavior 7 and 13 days after the implant surgery. One day after the last behavioral test, the animals were sacrificed and processed for nuclear exchange assays. This time frame was chosen to ensure that the miniature osmotic pumps would still be operating when the assays were performed, because the pumps were designed to run for 15 days. Moreover, two weeks of testosterone-treatment is enough time to observe the complete restoration of male copulatory behavior in Long-Evans rats.

Methods

The methods used for animal preparation, behavioral testing, data collection and analysis, and cell nuclear estrogen receptor assays were the same as described in the General Methods Chapter. Miniature osmotic pumps fitted to brain infusion cannulas were used to deliver fadrozole hydrochloride (CGS 16949A, Ciba-Geigy, Summit, NJ) into the right lateral ventricle for 14 days (see Appendix A, Figure 51 for a diagram of fadrozole's chemical structure). Fadrozole was first dissolved in ethanol and then diluted in either propylene glycol or PEG-200 (final ethanol concentration: 1.6%).

The animals were also implanted subcutaneously (ventral thorax) with combinations of either two, 10 mm Silastic capsules filled with crystalline 100% testosterone (Sigma Chemicals, St. Louis, MO; T, T/F, and T/F/E groups), and/or one, 5 mm Silastic capsule filled with crystalline 1% 17β-estradiol diluted in cholesterol (Sigma Chemicals, St. Louis, MO)(E and T/F/E groups). Gonadectomized male rats implanted with two, 10 mm testosterone filled Silastic capsules copulate normally, and have serum androgen levels similar to intact males (McGinnis et al. 1989). The Silastic capsules were prepared to restore comparable levels of nuclear androgen and estrogen receptor occupation to those found in intact adult male rats (McGinnis and Dreifuss 1989). For a negative control in the cell nuclear estrogen receptor and androgen receptor assays, gonadectomized male rats without Silastic capsules or intracranial implants were used (GdX group).

Results

Initially, pilot studies were conducted to determine the best dose of fadrozole to block the restoration of male copulatory behavior. The percentage of males ejaculating is shown in Table 9 ($\chi^2(3, N = 26) = 21.95$, $p < 0.0001$). The data for the percentage of males mounting and percentage of males intromitting were similar, so only the data for the

Table 9. Percentage of Males Ejaculating with Different Doses of Fadrozole

Group	Percentage of Males Ejaculating (test #2)	*n*	
Testosterone	92%	12	*
Testosterone & Fadrozole 1 µg/day ICV	100%	6	*
Testosterone & Fadrozole 20 µg/day ICV	0%	4	#
Testosterone & Fadrozole 100 µg/day ICV	0%	4	#

* $p < 0.05$, the observed proportion was significantly higher than the expected value
# $p < 0.05$, the observed proportion was significantly lower than the expected value

percentage of males ejaculating is shown. One µg/day fadrozole had no effect on the restoration of male copulatory behavior relative to testosterone. There was also no effect of one µg/day fadrozole on any individual measure of male copulatory behavior, such as mount frequency or ejaculation latency. Both 20 and 100 µg/day fadrozole completely blocked testosterone's restoration of male copulatory behavior. However, 100 µg/day fadrozole was not different from 20 µg/day fadrozole (i.e. none of the males copulated). Therefore, 20 µg/day fadrozole was used to complete the study.

Figure 18 shows the percentage of animals ejaculating on the postcastration test, test #1 (7 days after implant surgery), and test #2 (13 days after implant surgery). Chi-square tests indicated that following implant surgery, there were significant group differences in the percentage of animals ejaculating on test #1 ($\chi^2(3, N = 46) = 14.779, p = 0.0013$) and test #2 ($\chi^2(3, N = 46) = 28.863, p < 0.0001$). In animals implanted with two, 10 mm testosterone capsules and propylene glycol filled miniature osmotic pumps (T group), ejaculatory behavior was restored in 12 out of 16 animals (test #1) and 15 out of 16 animals (test #2). On the other hand, the majority of animals which received fadrozole treatment and testosterone capsules (T/F group) did not ejaculate during the tests 7 and 13 days following implant surgery (11 out of 14, and 12 out of 14 animals, respectively). Animals which received only one, 5 mm 1% estradiol capsule (E group) did not ejaculate over this time course (8 out of 10 animals for both tests). However, animals which

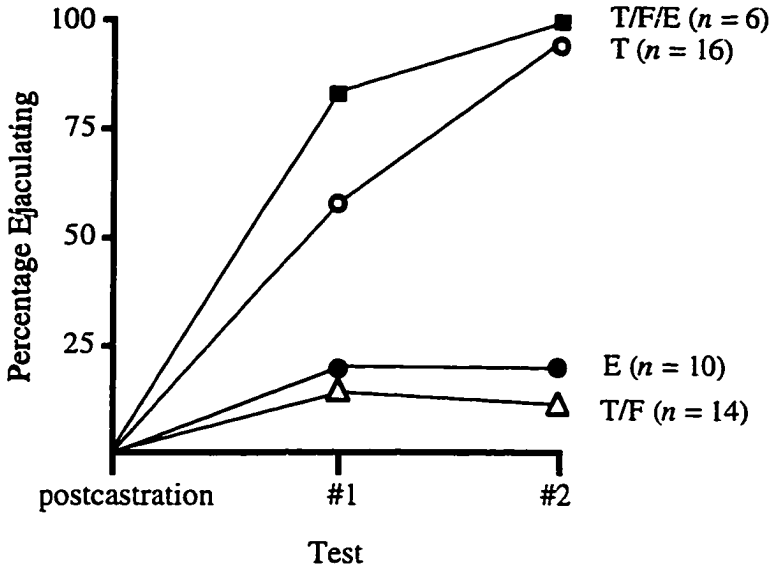


Figure 18. Percentage of Males Ejaculating

Restoration of male copulatory behavior measured by the percentage of animals ejaculating. Test #1 was 7 days after implant surgery and test #2 was 13 days after implant surgery. Abbreviations: T = testosterone; T/F = testosterone and 20 μg/day ICV fadrozole; T/F/E = testosterone, 20 μg/day ICV fadrozole, and estradiol; and E = estradiol. On test #1, the T/F and E groups were significantly different from the testosterone group (Fisher's Exact test, $p = 0.0038$ and $p = 0.0138$, respectively). On test #2, the T/F and E groups were significantly different from the testosterone group (Fisher's Exact test, $p < 0.0001$ and $p = 0.0002$, respectively). The T/F/E group was not significantly different from the T group on either test.

received fadrozole treatment with two, 10 mm testosterone capsules and one, 5 mm 1% estradiol capsule (T/F/E group) did ejaculate on test #1 (5 out of 6) and test #2 (6 out of 6). Post hoc cell contributions for test #1 showed that the proportion of animals ejaculating was significantly higher than the expected value in the T group ($p = 0.0071$), but less than the expected value for the T/F group ($p = 0.0117$). Post hoc cell contributions for test #2 showed that the proportions of animals ejaculating were significantly higher than the expected values in the T group ($p < 0.0001$) and the T/F/E group ($p = 0.0161$). However, the proportions of animals ejaculating in the T/F and E groups were significantly lower than the expected values ($p = 0.0003$ and $p = 0.0137$, respectively). The results for the percentage of animals mounting and the percentage of animals intromitting showed the same trends as the results for the percentage of animals ejaculating (data not shown).

Individual measures of male copulatory behavior were also recorded and analyzed in animals that copulated. Comparisons were made for raw measures such as mount frequency, intromission frequency, mount latency, intromission latency, ejaculation latency, and postejaculatory interval; and also for derived measures such as hit rate, intercopulatory interval, and copulatory duration. These data are tabulated in Appendix A, Table 16. The only animals in this study that were significantly different from the testosterone-treated gonadectomized males (T group) were estradiol-treated males (E group). Animals in the E group mounted significantly more than the T group on both test #1 (ANOVA F (3,19) = 13.596, $p < 0.0001$, mean ± SEM, 23 ± 3 vs. 8 ± 1, respectively) and test #2 (ANOVA F (3,22) = 14.749, $p < 0.0001$, mean ± SEM, 20 ± 5 vs. 7 ± 1, respectively). In addition, the hit rate was significantly lower in the E group on test #1 (ANOVA F (3,18) = 11.954, $p = 0.0002$, mean ± SEM, 36 ± 5 vs. 64 ± 3, respectively), because mount frequency was increased in the E group relative to the number of intromissions. The animals in the E group also took longer to copulate on test #2. This was indicated by significant increases in ejaculation latency (ANOVA F (3,22) = 5.701, p = 0.0048, mean ± SEM, 1022 ± 538 vs. 277 ± 52, respectively), intercopulatory interval (ANOVA F (3,22) = 8.049, $p = 0.0008$, mean ± SEM, 42 ± 11 vs. 18 ± 1, respectively), and copulatory duration (ANOVA F (3,22) = 8.193, $p = 0.0008$, mean ± SEM, 655 ± 192 vs. 194 ± 28, respectively). These results are in agreement with previous studies showing that treatment with estradiol alone results in impaired copulatory performance (Larsson et al. 1973b, Larsson et al. 1973a, Sodersten 1973, Beyer et al. 1981).

There was a significant interaction effect for partner preference (Figure 19, groups x test, repeated measures ANOVA F (6,66) = 4.109, p = 0.0015). Testosterone treatment significantly increased the amount of time spent with the receptive female relative to the non-receptive female in the T, T/F, and T/F/E groups, but not in the E group (Figure 19). Repeated measures contrasts on the group x test interaction indicated that the E group was significantly different (ANOVA F (2,66) = 3.662, p = 0.0310) from the T group.

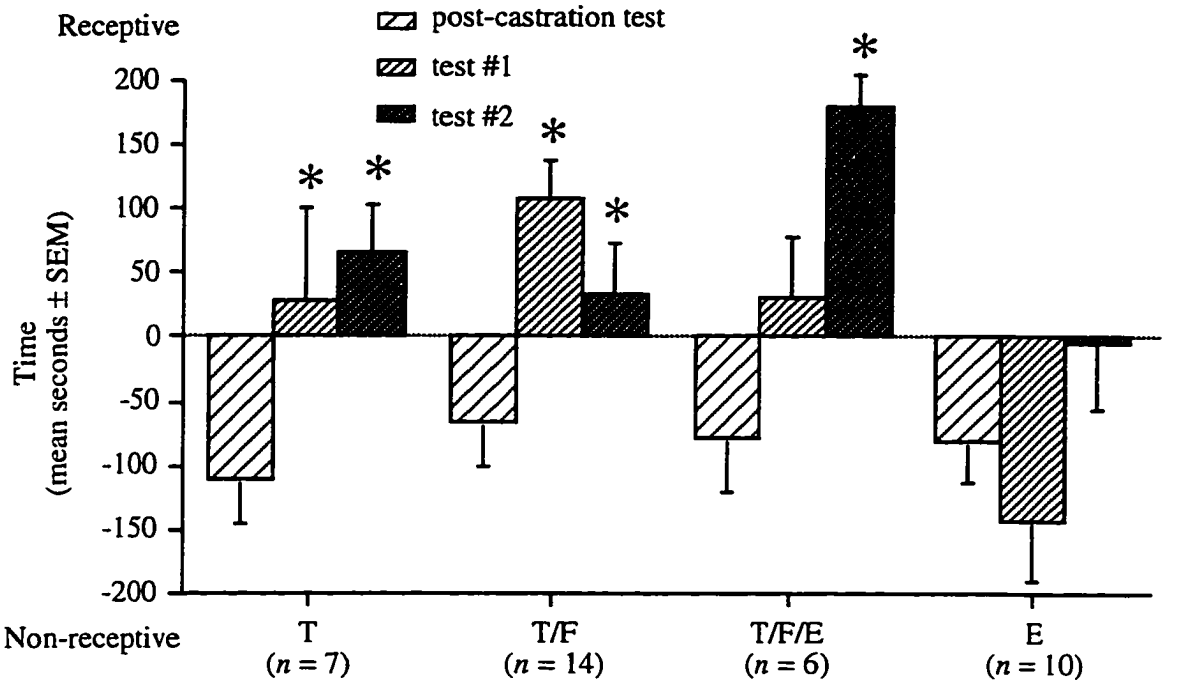


Figure 19. Partner Preference

Partner preference test (test #1, 7 days post-surgery; test #2, 13 days post-surgery)(T = testosterone; T/F = testosterone and fadrozole; T/F/E = testosterone, fadrozole, and estradiol; and E = estradiol). The y-axis depicts time spent with the non-receptive female subtracted from time spent with the receptive female. Positive values indicate that more time was spent with the receptive female and vice versa. (* $p < 0.05$, within group comparison vs. pretest, Least Square Mean Test)

However, both the T/F (ANOVA F (2,66) = 1.321, p = 0.2738,) and T/F/E (ANOVA F (2,66) = 0.913, p = 0.4064) groups were not significantly different from the T group. In addition, the number of times the male crossed a chamber boundary was recorded as a measure of the animal's activity. There was no effect for boundary crossing frequency (ANOVA F (6,66) =0.775, p = 0.5924, Appendix A, Figure 52) or for time spent in the empty chamber (ANOVA F (6,66) = 0.578, p = 0.7469, Appendix A, Figure 53).

To determine whether fadrozole blocked brain aromatase, and thus the metabolic conversion of testosterone to estradiol, I measured brain (pooled hypothalamus, preoptic area, septum, and amygdala) nuclear estrogen receptor occupation with in vitro cell nuclear exchange assays (Figure 20, ANOVA F (4,24) = 10.07, p = 0.0001). Gonadectomized males, without testosterone capsules (GdX group), were used as a negative control to

show that in the absence of testosterone, brain nuclear estrogen receptor occupation was significantly lower than testosterone-treated males (Dunnett's one-tailed post hoc test, GdX vs. T, $p < 0.05$). Fadrozole treatment to gonadectomized males implanted with testosterone capsules (T/F group) resulted in a 59% decrease in brain nuclear estrogen receptor occupation relative to gonadectomized males implanted with testosterone capsules and propylene glycol filled miniature osmotic pumps (T group, miniature osmotic pump vehicle control, Dunnett's one-tailed post hoc test, F/T vs. T, $p < 0.05$). Both the E and T/F/E groups were not significantly different from the T group (Dunnett's two-tailed post hoc test, E or T/F/E vs. T, $p > 0.05$). This indicates that one, 5 mm 1% estradiol capsule is appropriate to replicate the effects of estradiol derived from testosterone aromatization in animals implanted with two, 10 mm 100% testosterone capsules.

The McGinnis lab has previously shown that one, 5 mm 1% estradiol capsule does not alter nuclear androgen receptor occupation in the brain (McGinnis and Dreifuss 1989). Nuclear androgen receptor occupation was measured to determine if fadrozole blocked brain (pooled hypothalamus, preoptic area, septum, and amygdala) androgen receptors (Figure 21, ANOVA $F(2,21) = 12.21$, $p = 0.0003$). In the absence of testosterone (GdX group), nuclear androgen receptor occupation levels were significantly lower than those in both the T and T/F groups (Scheffe's S post hoc test, GdX vs. T, $p < 0.05$). However, there was no significant effect on brain androgen receptor occupation in animals implanted with testosterone capsules and fadrozole filled miniature osmotic pumps. In addition, fadrozole did not significantly reduce seminal vesicle weights (Appendix A, Figure 64). This indicates that ICV administration of fadrozole did not block peripheral androgen receptors.

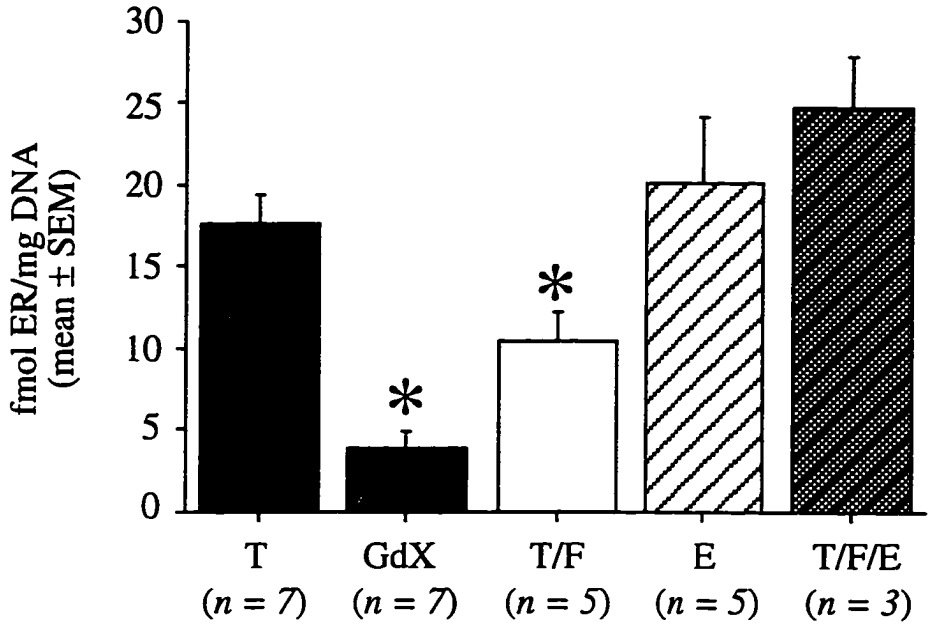


Figure 20. Cell Nuclear Estrogen Receptor Occupation

Nuclear estrogen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala)(T = gonadectomized male implanted with testosterone capsules and a miniature osmotic pump filled with propylene glycol; GdX = gonadectomized male, T/F = gonadectomized male implanted with testosterone capsules and a miniature osmotic pump filled with fadrozole, E = gonadectomized male implanted with an estradiol capsule, and T/F/E = gonadectomized male implanted with testosterone capsules, an estradiol capsule, and a miniature osmotic pump filled with fadrozole). Chronic intracerebroventricular administration of 20 μg/day fadrozole significantly decreased cell nuclear estrogen receptor occupation (* $p < 0.05$ vs. T, Dunnett's one-tailed post-hoc test).

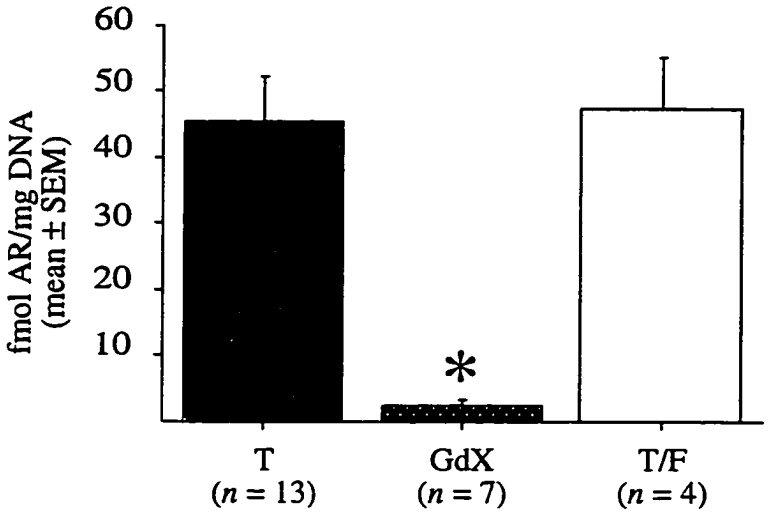


Figure 21. Cell Nuclear Androgen Receptor Occupation

Nuclear androgen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala)(T = gonadectomized male implanted with testosterone capsules and a miniature osmotic pump filled with propylene glycol; GdX = gonadectomized male, T/F = gonadectomized male implanted with testosterone capsules and a miniature osmotic pump filled with fadrozole). Chronic intracerebroventricular administration of 20 μg/day fadrozole did not affect brain cell nuclear androgen receptor occupation (* $p <$ 0.05 vs. T, Dunnett's two-tailed post-hoc test).

Discussion

The role of aromatization in the restoration of male copulatory behavior and partner preference was assessed by chronic infusion of the aromatase inhibitor, fadrozole, into the right lateral ventricle. I found that 20 μg/day of fadrozole completely blocked testosterone's restoration of male copulatory behavior (T/F group). After two weeks of treatment, male copulatory behavior was completely restored in animals implanted with testosterone capsules and receiving propylene glycol (miniature osmotic pump vehicle control) into the lateral ventricle (T group). This shows that the infusion procedure did not adversely affect copulatory behavior in adult male rats. To determine if estradiol acts synergistically with testosterone to reestablish male copulatory behavior in non-behaving gonadectomized rats, one group of animals received testosterone, fadrozole, and estradiol (T/F/E group). These rats copulated normally, suggesting that the loss of male copulatory behavior in the T/F group was due to a decrease in estradiol stimulation resulting from fadrozole's inhibition of brain aromatase. Another group received one, 5 mm 1% estradiol capsule without testosterone or fadrozole treatment (E group). The results from this group clearly show that a physiological estradiol dose by itself cannot restore male copulatory behavior. I am using the phrase physiological estradiol dose here because I wish to stress the importance of how much estradiol was given. It is important to stress that what is considered a physiological estradiol dose in a proestrus female rat (McGinnis et al. 1981) is considerably higher than a physiological estradiol dose in a male rat (McGinnis and Dreifuss 1989) as measured by cell nuclear exchange assays. The cell nuclear estrogen receptor exchange assays show that implanting one, 5 mm 1% estradiol capsule results in the same level of nuclear estrogen receptor occupation as found in animals implanted with two, 10 mm testosterone filled Silastic capsules or gonadally intact males (McGinnis and Dreifuss 1989). Therefore, this is a physiologically relevant dose of estradiol to give to adult male rats. This experiment is

important because it demonstrates that when brain nuclear estrogen receptor occupation is at gonadally intact levels, both testosterone and estradiol are necessary for the expression of male copulatory behavior, and that the restoration of male copulatory behavior is not solely the result of testosterone's aromatization to estradiol.

I found that fadrozole completely blocked testosterone's restoration of male copulatory behavior. After two weeks of treatment, 89% of the animals failed to either mount, intromit, or ejaculate. Of those few males who did copulate, fadrozole did not adversely affect any individual parameter of male copulatory behavior. Previous studies testing the effects of fadrozole on male copulatory behavior have employed maintenance paradigms and have used timed copulatory behavior tests (Bonsall et al. 1992, Clancy et al. 1995). These methodological differences make it difficult to directly compare my results with theirs. However, a general comparison points out one interesting facet. These other studies have shown that fadrozole treatment impairs the maintenance of male copulatory behavior by affecting individual aspects of copulation (mount, intromission, and ejaculation frequency and latency), rather than completely blocking all copulatory behavior as I have shown here. Therefore, it seems that the restoration of male copulatory behavior is more sensitive to fadrozole treatment than the maintenance of male copulatory behavior. These results agree with prior work showing that it is more difficult to block the maintenance of male copulatory behavior relative to blocking the restoration of male copulatory behavior (Damassa et al. 1977, McGinnis and Mirth 1986).

Partner preference tests have been previously used as an index of sexual motivation (Everitt 1990). Gonadectomized male rats have been shown to lose their preference for sexually receptive females over non-receptive females (Merkx 1984). Animals receiving either testosterone or testosterone and fadrozole treatment spent more time with sexually receptive females following 13 days of treatment. However, animals receiving estradiol treatment did not show a significant preference for sexually receptive females. These results suggest that partner preference is not dependent on estradiol, but requires testosterone. My

results differ from work showing that estradiol can either maintain or restore partner preference in gonadectomized male rats (Merkx 1984, Bakker et al. 1993). However, the Silastic capsules used in these other studies delivered more than fifty times the dose of estradiol I used. Therefore, differences in estradiol doses between these studies may account for the discrepancy in estradiol's role in the expression of partner preference. The notion that high and low doses of estradiol could have differential effects on the restoration of partner preference is supported by studies showing that male copulatory behavior can be restored by doses of estradiol which exceed those found in intact male rats (Davidson 1969, Sodersten 1973, Krey et al. 1980).

Another aspect of this study was to determine the effects of fadrozole on estrogen and androgen receptor binding. I used a cell nuclear estrogen receptor exchange assay to show that fadrozole significantly decreased brain estrogen receptor occupation, presumably by reducing the amount of estradiol available. It was also essential to confirm that fadrozole did not affect brain androgen receptor occupation, because this could have accounted for its effects on male copulatory behavior. Work showing that the aromatase inhibitor, androst-1,4,6-triene-3,17-dione (ATD), blocks male copulatory behavior provided strong evidence supporting the aromatization hypothesis (Christensen and Clemens 1975, Beyer et al. 1976b, Morali et al. 1977, Sodersten et al. 1986). However, ATD has also been found to block androgen receptors (Debold et al. 1981, Slama et al. 1986, Kaplan and McGinnis 1989, Summerfield et al. 1995). Therefore, ATD's effects on male sex behavior could be attributed not only to its inhibition of aromatase, but also to its inhibition of androgen receptors. My results agree with other studies showing that fadrozole does not affect androgen receptors (Bonsall et al. 1992). These data provide strong support for the hypothesis that fadrozole's blockade of the restoration of male copulatory behavior is mediated by inhibition of brain aromatase and not inhibition of androgen receptors.

My work implies that the mechanisms responsible for the restoration of partner preference and male copulatory behavior are different. Animals treated with fadrozole via

the lateral ventricle are as active as control males and are sexually motivated, but do not show any copulatory behavior. My work suggests that without aromatization, testosterone cannot restore male copulatory behavior, but it can restore partner preference. However, systemic estradiol treatment, at a dose which provides physiologically relevant nuclear estrogen receptor occupation levels, is not sufficient to restore male copulatory behavior or partner preference. Thus, my work supports the notion that distinct neural mechanisms could mediate copulatory performance and sexual motivation. These data provide evidence against a strict interpretation of the aromatization hypothesis, and favor a modified version stating that male copulatory behavior is mediated by both testosterone and estradiol (Perez Palacios et al. 1975, Beyer et al. 1976a, Sodersten 1979).

Chapter 5 - The Role of Estrogen Receptor Action in the Restoration of Hormone-Dependent Sociosexual Behaviors

The results from the fadrozole study (previous chapter) clearly indicate a role for estradiol in the restoration of male copulatory behavior. This is based on fact that blocking testosterone's aromatization blocks the restoration of male copulatory behavior. If estradiol has a role in mediating male copulatory behavior, are cell nuclear estrogen receptors involved? The concept that the behavioral effects of estradiol are primarily mediated via classical nuclear estrogen receptor action in male rats is widely accepted (reviewed in Yahr 1979, McCarthy 1994, Handa et al. 1996). The prevailing dogma is that steroid hormones typically enter target cells and bind specific intracellular receptors. This activates the steroid receptors and leads to the alteration of genomic expression (Mooradian et al. 1987, Berger and Watson 1989), which in turn, influences neuronal processing and ultimately produces changes in copulatory behavior (McEwen 1988, Rose 1990). According to this hypothesis, estradiol's role in the restoration of male copulatory behavior should occur through the activation of cell nuclear estrogen receptors.

A common approach to address the role of estrogen receptor action has been to use estrogen receptor antagonists (antiestrogens) to block the activation of nuclear estrogen receptors. However, the few studies that have been performed concerning the effects of antiestrogens on male copulatory behavior (reviewed in the introduction on page 61, and listed in Table 10) have not definitively shown that antiestrogens blocked male copulatory behavior. A major problem with these studies has been the lack of selective estrogen receptor antagonists that did not have significant estrogenic activity (Landau 1986). Therefore, the weak effects of antiestrogens, such as tamoxifen or CI-628, on male

Table 10. Survey of the Effects of Antiestrogens on Male Copulatory Behavior in Rats

Antiestrogen	Behavior Blocked?	Study
MER-25	no	(Luttge 1975)
	no	(Baum and Vreeburg 1976)
	no	(Beyer et al. 1976b)
CI-628	yes	(Luttge 1975)
	no	(Yahr and Gerling 1978)
cis-clomifene	no	(Beyer et al. 1976b)
tamoxifen	yes	(Beyer et al. 1976b)
hydroxytamoxifen	no	(McGinnis et al. 1990)

Note: The criteria used to compare the effectiveness of the antiestrogens used in these studies was based on whether or not the antiestrogen blocked the effect of testosterone on ejaculatory behavior.

copulatory behavior could have been due to technical problems, because these compounds are partial agonists.

Recently, "pure" steroidal estrogen receptor antagonists have been developed that do not possess any partial agonist activity (Poulin et al. 1989, Kangas 1992, Wakeling 1992). I thought that using these new "pure" antiestrogens was a way to finally test if nuclear estrogen receptor action was directly involved in mediating male copulatory behavior. The characteristics of several "pure" estrogen receptor antagonists (EM-219 (Labrie et al. 1992), raloxifene (Neubauer et al. 1993), ICI 164384 (Bondy and Zacharewski 1993), and ICI 182780 (Parker 1993a)) have been published. However, it has been subsequently shown that the nonsteroidal "pure" antiestrogens, EM-219 (Li et al. 1995) and raloxifene (Draper et al. 1995), are partial agonists. Zeneca Pharmaceuticals' antiestrogens (formerly Imperial Chemical Industries [ICI]), ICI 164384 and ICI 182780, are some of the most selective "pure" estrogen receptor antagonists developed to date (Nicholson et al. 1996). None of these "pure" antiestrogens have been tested for their ability to disrupt masculine copulatory behavior in rats.

When this project was first proposed, I had the naive idea that I would simply start off by addressing the role of estrogen receptor action in male copulatory behavior. I was

not the first to address this question (and probably not the last), but I thought I had a few advantages that would allow me to study the behavioral aspects of this problem in a relatively short period of time. The concept was fairly simple: to use a well established estrogen receptor antagonist to assess the role of estrogen receptor action in the restoration of male rat copulatory behavior. I thought that because the antagonist I originally proposed to work with (ICI 164384) had been shown to be more effective than any of the previously used antiestrogens, combined with a conservative experimental design, the proposed experiment was almost certain to resolve this controversy. However, this was not the case, and this chapter has been subdivided into four parts to show how this work progressed.

The first part describes my work with the antiestrogen ICI 182780 on male copulatory behavior. Intracerebroventricular administration (ICV) of ICI 182780 had no effect on either male copulatory behavior or brain cell nuclear estrogen receptor binding. Unfortunately, ICI 182780 did not block brain cell nuclear estrogen receptor binding, and this was a major problem, because I did not know if the nuclear exchange assay was the problem or not. Due to the problems of studying estrogen receptor action in male rats (specifically, the extremely low level of peripheral estrogen receptors combined with the use of a drug that could not cross the blood-brain barrier), I temporarily switched over to working with female rats because they express higher levels of peripheral estrogen receptors compared to male rats. This allowed me to go from using two animals per data point with the males to using only one animal per data point with the females. In addition, I was also able to use fewer data points, because there was much lower interanimal variability in the females. The second part of this chapter describes my work on the effects of ICI 182780 on cell nuclear estrogen receptor binding in female rats.

ICI 182780 did not affect cell nuclear estrogen receptor binding in female rats either. After investing a lot of time working with ICI 182780 and getting poor results, I decided to use a different "pure" antiestrogen, RU 58668. However, much less was known about RU 58668 than ICI 182780, so a series of pilot studies in female rats were

performed to determine the effectiveness of RU 58668 on blocking several classic estrogen-dependent behaviors and biochemical measures. Female rats were chosen because these studies could be performed much faster and more economically in female rats than in males, but more importantly, there also was a large body of literature in female rats on the effects of other antiestrogens to compare my results with. Therefore, by working with female rats before the males, I would know if RU 58668 was going to be a suitable antiestrogen. The third part of this chapter details these studies with RU 58668 in female rats. My results showed that RU 58668 could block brain estrogen receptor action, measured with a nuclear exchange assay, and also block estrogen-dependent behaviors in female rats (i.e., female copulatory behavior and feeding behavior). Finally, the fourth part of this chapter describes my work with RU 58668 in examining the role of estrogen receptor action on copulatory behavior in male rats. My results showed that RU 58668 did not affect male copulatory behavior, but I was able to show that RU 58668 did reduce brain cell nuclear estrogen receptor binding to the level found in gonadectomized males. Therefore, my results indicate that brain cell nuclear estrogen receptor action might not be involved in the restoration of male copulatory behavior.

Part 1: Effects of the Antiestrogen ICI 182780 on Male Copulatory Behavior

Introduction

The steroidal antiestrogen ICI 164384, a 7α-alkylamide derivative of 17β-estradiol, was the first antiestrogen to be developed that was not a partial agonist (Wakeling and Bowler 1988). For example, ICI 164384 did not stimulate uterine growth in immature female rats, which was in contrast to other more established antiestrogens, such as tamoxifen. ICI 164384 was also found to have a high affinity for human uterine estrogen receptors, with a K_D of 0.69 nM (Weatherill et al. 1988). Later, Zeneca found that a similar compound, ICI 182780, was more potent in vivo and focused its development efforts into this antiestrogen (Wakeling and Bowler 1992). The structural difference between ICI 164384 and ICI 182780 was that the 7α-side chain of ICI 16384's amide group was replaced by a sulfinyl group, and the last alkyl group was fluorinated. A diagram of ICI 182780's chemical structure is provided in Appendix A, Figure 51. These modifications resulted in a compound with a 4-5 fold greater affinity for estrogen receptors than ICI 164384 (Wakeling 1993). Therefore, I started working with ICI 182780 because (1) it was a more effective antiestrogen and (2) ICI 164384 was taken out of production.

Experimental Design

The experimental design for this experiment was identical to the fadrozole pilot study discussed in the last chapter. The only difference was that instead of the aromatase inhibitor fadrozole, the antiestrogen ICI 182780 was used. In fact, the reason the fadrozole was delivered by the intracerebroventricular route (ICV) was because ICI 182780 could not cross the blood-brain barrier (Wade et al. 1993a), and I wanted to be able to compare

the effects of different inhibitors on male copulatory behavior under the same experimental conditions. Because this was a pilot study, only one male copulatory behavior test was given after two weeks of testosterone/antiestrogen treatment. At the time this study was performed, I was not doing partner preference tests or 50 kHz ultrasonic vocalization/scent marking tests. One day after the behavior test, cell nuclear estrogen receptor assays were performed to assess the effects of ICI 182780.

Methods

The methods used for animal preparation, behavioral testing, data collection and analysis, and cell nuclear estrogen receptor assays were the same as described in the General Methods Chapter. Miniature osmotic pumps fitted to brain infusion cannulas were used to deliver ICI 182780 (Zeneca Pharmaceuticals, Macclesfield, UK) into the right lateral ventricle for 14 days. The ICI 182780 was dissolved directly in propylene glycol. Control males were gonadectomized and implanted with two, 10 mm testosterone-filled Silastic capsules and miniature osmotic pumps filled with propylene glycol that were connected to a cannula in the right lateral ventricle.

Results

ICV administration of the antiestrogen ICI 182780 did not have any effect on testosterone's restoration of male copulatory behavior ($\chi^2(3, N = 26) = 1.507$, $p = 0.6806$). Table 11 displays data for the percentage of males ejaculating after two weeks of ICI 182780 treatment. The data for the percentage of males mounting and the percentage of males intromitting were identical to the percentage of males ejaculating, so these results were not shown. Analysis of individual measures of male copulatory behavior did not reveal any significant trends that were dose-dependent (Appendix A, Table 18).

Table 11. Percentage of Males Ejaculating with Different Doses of ICI 182780

Group	Percentage of Males Ejaculating (test #2)	*n*
Testosterone	92%	12
Testosterone & ICI 182780 10 μg/day ICV	83%	6
Testosterone & ICI 182780 100 μg/day ICV	100%	4
Testosterone & ICI 182780 500 μg/day ICV	75%	4

Cell nuclear estrogen receptor assays were performed to determine if the behavioral results correlated with blocking brain estrogen receptors. Figure 22 shows the effects of three doses of ICI 182780 on brain (pooled amygdala, hypothalamus, preoptic area, and septum; ANOVA F (4,17) = 14.42, $p < 0.0001$) and pituitary (ANOVA F (4,17) = 8.16, $p < 0.0001$) cell nuclear estrogen receptor binding. ICI 182780 did not have any significant effect in the brain or pituitary. However, gonadectomized males (GdX group) had significantly lower levels of cell nuclear estrogen receptors in both the brain and pituitary relative to testosterone-treated gonadectomized males (T group).

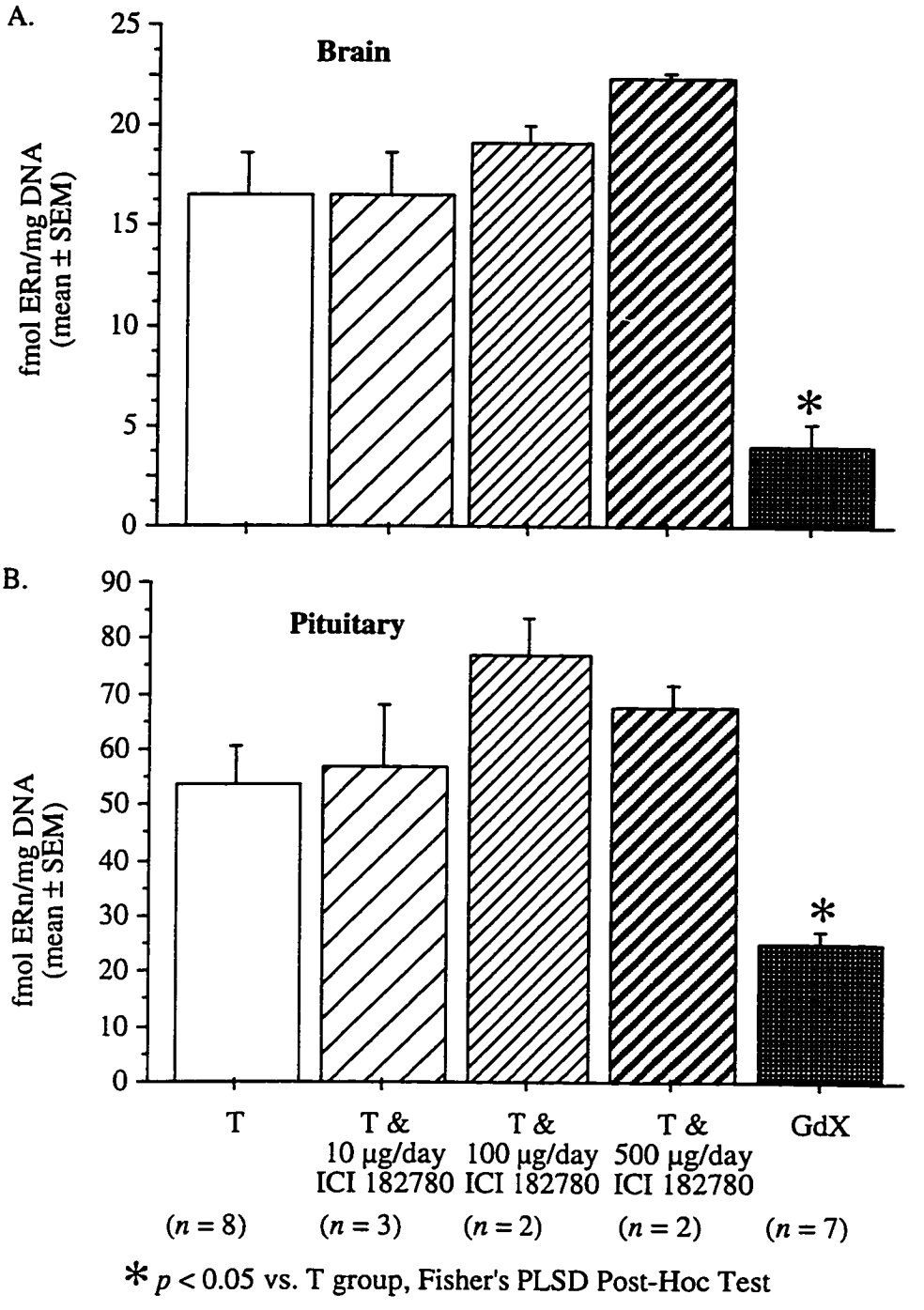


Figure 22. The Effects of ICI 182780 on Cell Nuclear Estrogen Receptor Binding in Male Rats

* $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

Discussion

The effects of ICI 182780 on female copulatory behavior have been assessed in both rats (Wade et al. 1993a) and hamsters (Wade et al. 1993b), but this antiestrogen did not block estradiol's effects on female copulatory behavior as well as other antiestrogens have in the past (Morin et al. 1976, Wade and Blaustein 1978). This result was surprising because ICI 182780 was said to be a much better estrogen receptor antagonist, based on its high affinity for estrogen receptors and its lack of intrinsic estrogenic activity (Wakeling and Bowler 1992). However, it was found that ICI 182780 did not block brain estrogen receptors when it was injected subcutaneously (Wade et al. 1993a), and this explained the compound's weak effects on female copulatory behavior. On the other hand, ICI 182780 could compete in vitro for radiolabeled estradiol binding as well as unlabeled estradiol in cytosolic fractions prepared from female rat brains. Therefore, the problem was that ICI 182780 did not pass through the blood-brain barrier, and not that ICI 182780 could not compete with estradiol for brain estrogen receptors. Accordingly, I used intracerebroventricular (ICV) drug delivery in the my experiment to circumvent this problem.

Even though ICI 182780 did not affect male copulatory behavior, only limited conclusions from this study can be made about the role of estrogen receptors in male copulatory behavior, because ICI 182780 did not affect brain cell nuclear estrogen binding. Showing that ICI 182780 blocked brain estrogen receptors was a crucial part of this study because knowing whether the antiestrogen blocked brain estrogen receptors was central to determining the role of nuclear estrogen receptors in male copulatory behavior. Without this knowledge, it was impossible to know if the antiestrogen was effective. This was a major shortcoming in the other studies that have tested the effects of antiestrogens on male copulatory behavior, because these studies did not have any controls for whether their

antiestrogens worked. Therefore, the results of this study cannot be used to support the hypothesis that brain nuclear estrogen receptors are, or are not, involved in male copulatory behavior.

Part 2: Effects of the Antiestrogen ICI 182780 on Cell Nuclear Estrogen Receptor Binding in Female Rats

Introduction

Since I was unable to show that ICV administration of ICI 182780 blocked either male copulatory behavior or brain cell nuclear estrogen receptor binding, the question of whether this antiestrogen was really effective came up, and specifically, whether ICI 182780 would reduce the level of cell nuclear estrogen receptor binding measured in a nuclear exchange assay. There was a considerable body of literature indicating that ICI 182780 was indeed an effective antiestrogen (reviewed in Nicholson et al. 1996). Therefore, I decided to test if ICI 182780 blocked cell nuclear estrogen receptor binding in female rats. I choose female rats because they express higher levels of estrogen receptors than male rats (Kranzler et al. 1984, Lauber et al. 1991a), and previous studies testing the effects of other antiestrogens with nuclear exchange assays used female rats (Roy 1978). Both tamoxifen and CI-628 acted as agonists, rather than inhibiting the exchange of radiolabeled estradiol, in other studies that used cell nuclear exchange assays. However, I hypothesized that because ICI 182780 was a steroidal antiestrogen and not a partial agonist, it might behave differently from the nonsteroidal antiestrogens in this assay.

Experimental Design

An in vitro cell nuclear exchange assay was used to determine if injections of ICI 182780 reduced cell nuclear ^{3}H-estradiol binding in individual ovariectomized adult female rats (McGinnis et al. 1981). All animals were injected with a saturating dose (Anderson et al. 1972) of unlabeled estradiol 2 hours before the assay (2.7 μg/kg sc), except for the ovariectomized females (OvX group), to activate the estrogen receptors. In

addition, two groups received a single 1 or 5 mg/kg injection of ICI 182780 2 hours before the assay, along with the unlabeled estradiol.

Methods

The method used for the cell nuclear estrogen receptor assay was the same as was outlined in the General Methods Chapter except for the following changes. Individual animals were used instead of pooling tissue from two rats, because females express higher levels of nuclear estrogen receptors than male rats. Three tissues were taken: brain (pooled amygdala, hypothalamus, preoptic area, and septum), pituitary, and uterus. Uterine tissue was homogenized with a Tekmar Tissuemizer set at 50% power for 15 seconds while in an ice bath, because of this tissue's fibrous nature.

Results

The effect of ICI 182780 on cell nuclear estrogen receptor binding in the female rat brain (ANOVA F (3,6) = 27.59, p = 0.0007), pituitary (ANOVA F (3,6) = 12.55, p = 0.0054), and uterus (ANOVA F (3,8) = 41.12, $p < 0.0001$) are shown in Figure 23A-C, respectively. Ovariectomized females (OvX group) had significantly lower ($p < 0.001$) levels of cell nuclear estrogen receptor binding in all three tissues than ovariectomized females injected with estradiol (E_2 group). Neither 1 mg/kg nor 5 mg/kg reduced the level of cell nuclear estrogen receptor binding in any tissue. Surprisingly, the level of brain cell nuclear estrogen receptor binding was significantly higher (p = 0.0123) in the "E_2 & 5 mg/kg "group. However, this result should be viewed with caution because there was a n of only 2 in this group, and also because ICI 182780 did not affect the pituitary or uterus.

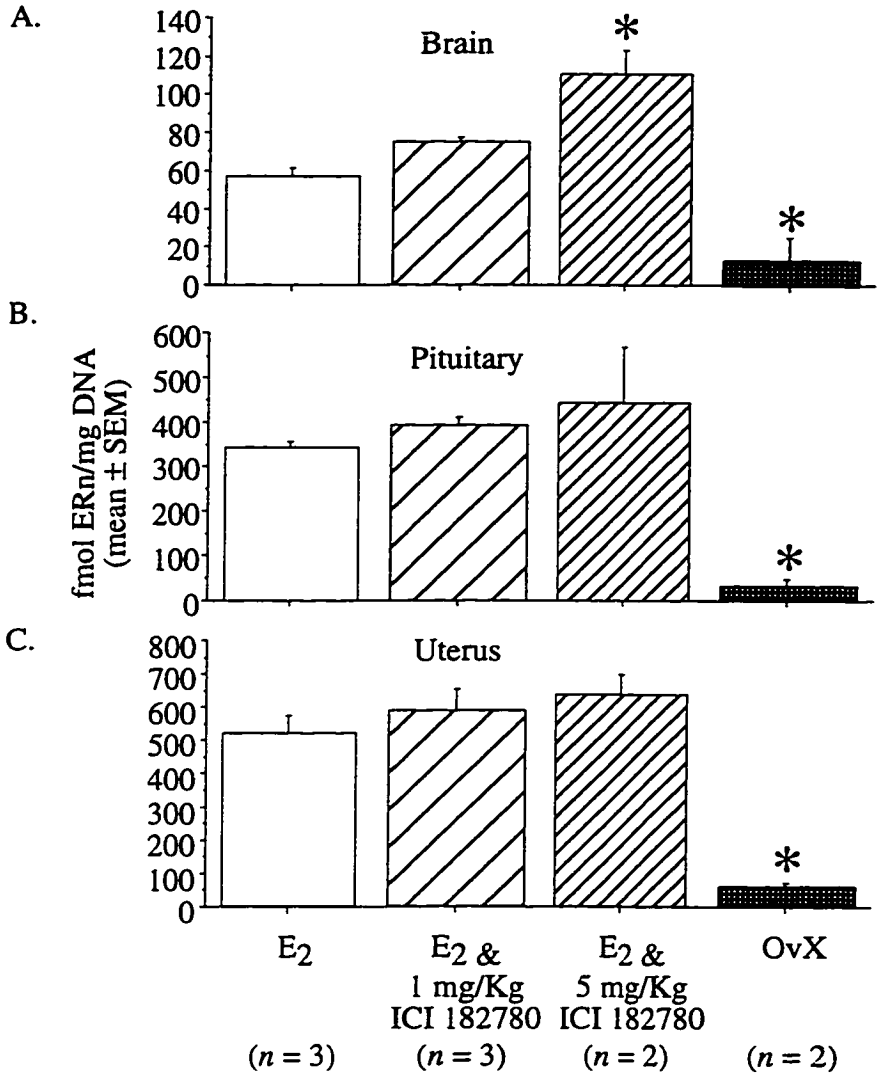


Figure 23. The Effects of ICI 182780 on Cell Nuclear Estrogen Receptor Binding in Female Rats

* $p < 0.05$ vs. T; Fisher's PLSD Test

Discussion

The purpose of this experiment was to test whether ICI 182780 would reduce cell nuclear estrogen receptor binding in female rats, because this antiestrogen did not reduce cell nuclear estrogen receptor binding in male rats. The reason female rats were used in this experiment was because they express much higher levels of peripheral estrogen receptors

(in the uterus and pituitary) than male rats. Therefore, ICV administration was not necessary. The use of female rats also allowed us to compare our results with those of other laboratories, since similar studies have not been performed with antiestrogens in male rats.

Considering the fact that other antiestrogens have acted as agonists in nuclear exchange assays (Roy 1978), why was ICI 182780 hypothesized to be different? First, ICI 164384 (which is similar to ICI 182780) had been previously shown to reduce mouse uterine nuclear estrogen receptor binding in as little as 30 minutes after it was injected (Gibson et al. 1991). However, the nuclear exchange assay used by Gibson et al. was notably different from the one used here (McGinnis et al. 1981). Therefore, the differences in the buffer salt concentration, the incubation duration and temperature, and the separation method could have been responsible for the opposing outcomes in our assay results. The second reason why ICI 182780 was hypothesized to reduce cell nuclear estrogen receptor binding was based upon its proposed mechanism of action. For example, several studies have shown that treatment with ICI 164384 results in the degradation of uterine estrogen receptors (Gibson et al. 1991). The mechanisms for this antiestrogen-induced loss of estrogen receptors include: impaired receptor dimerization and increased protein turnover (Dauvois et al. 1992), and inhibition of nuclear transport (Dauvois et al. 1993, Miksicek et al. 1995). So, treatment with ICI 182780 should have reduced the level of nuclear estrogen receptor binding in spite of the fact that the assay methodology was different from the one used by Gibson et al (1991).

There is no simple explanation for why ICI 182780 did not reduce brain cell nuclear estrogen receptor binding. One possibility is that the ICI 182780 sent to the laboratory from Zeneca Pharmaceuticals was defective. However, several different shipments of the antiestrogen were used in this and the previous study because we received them in 50 mg aliquots. Therefore, it seems unlikely that several shipments were all defective. Another possibility that could explain why ICI 182780 did not reduce uterine

and pituitary cell nuclear estrogen receptor binding in the females was that it might not have had enough time to reach the tissues, because the assay was performed 2 hours after the antiestrogen was subcutaneously injected (Gibson et al. (1991) used intraperitoneal (IP) injections). Moreover, data presented in the next section of this chapter on the effects of the antiestrogen, RU 58668, on cell nuclear estrogen receptor binding supports the hypothesis that it takes at least several hours after a subcutaneous injection of a "pure antiestrogen" to reduce cell nuclear estrogen receptor binding.

In conclusion, ICI 182780 did not reduce the level of cell nuclear estrogen receptor binding in female (this section) or male (previous section) rats. However, it is not known if ICI 182780 affected other estrogen-dependent measures. One control that could have been performed would have been to measure the level of cytosolic progestin receptors in the females (because progestin receptors are induced by estrogen). This would have allowed us to determine if there were technical problems with the antiestrogen. On the other hand, there are no well established estrogen-dependent biochemical measures in male rats that are not also affected by androgens. Therefore, because I was unable to determine the effectiveness of ICI 182780 with the nuclear exchange assay, coupled with the fact that the drug was in short supply, I began working with another pure antiestrogen from Roussel UCLAF, RU 58668. However, because little was known about RU 58668, I wanted to establish whether this antiestrogen was effective in female rats before using it in male rats. The effect of RU 58668 on behavioral and biochemical measures of estrogen receptor action are presented in the following section.

Part 3: Tests of the Antiestrogen RU 58668 in Female Rats: A Validation of this New Antiestrogen

Introduction

Many estrogen receptor antagonists have been developed since the introduction of MER-25 (ethamoxytriphetol), the first antiestrogen (Lerner et al. 1958). However, the great majority of these compounds, such as tamoxifen (ICI 46474) and CI-628 (nitromifene), exhibit complicated agonist/antagonist properties depending upon the dose administered and the tissue system studied (Jordan 1984). While they act as antiestrogens by blocking female sexual behavior (Morin et al. 1976, Roy and Wade 1977, Meisel et al. 1987), they act as estrogens by reducing food intake and body weight (Roy and Wade 1976, Roy et al. 1977, Wade and Blaustein 1978, Wade and Heller 1993). These nonsteroidal compounds have been recently designated as type I antiestrogens because they are all are partial agonists (Hedden et al. 1995).

Type II or "pure" antiestrogens are compounds which do not possess agonistic properties. ICI 164384 was the first "pure" steroidal antiestrogen to be developed (Wakeling and Bowler 1988). ICI 182780 is a close relative to ICI 164384, but is more potent in vivo (Wakeling and Bowler 1992). Only ICI 182780 has been tested on sexual behavior. In rats, ICI 182780 suppressed female sexual behavior, but only after long term exposure (24 days). Furthermore, it did not reduce food intake or body weight (Wade et al. 1993a). In Syrian hamsters, ICI 182780 blocked female sexual behavior after 2 days of exposure, but did not reduce food intake over 1 month of treatment (Wade et al. 1993b). However, ICI 182780 did attenuate the effects of estradiol benzoate on body weight in female hamsters (Wade et al. 1993b). In addition, ICI 182780 treatment did not affect plasma luteinizing hormone levels (Wakeling and Bowler 1988, Thomas et al. 1994). It has

been proposed that ICI 182780 might not cross the blood-brain barrier, and that these complex effects are due to blockade of peripheral estrogen receptor action (Wakeling and Bowler 1992, Wade et al. 1993a). However, ICI 182780 can compete for rat brain estrogen receptors in vitro (Wade et al. 1993a).

The compound utilized in this study, RU 58668, is a steroidal type II antiestrogen believed to be devoid of any partial agonist activity (Van de Velde et al. 1994, Nique and Van de Velde 1995). It differs from ICI 182780 in that its bulky side chain extends from estradiol's 11β carbon rather than from estradiol's 7α carbon. RU 58668 displays an affinity for estrogen receptors similar to 4-OH-tamoxifen, but is less selective in its binding to other steroid hormone receptors (Van de Velde et al. 1994). While tamoxifen and ICI 182780 do not bind glucocorticoid or progesterone receptors in vitro, RU 58668 does. However, results from in vivo bioassays for progesterone-induced endometrial proliferation and glucocorticoid-induced thymolysis indicate that RU 58668 is neither a progesterone/glucocorticoid receptor agonist nor antagonist (Van de Velde et al. 1994). Both RU 58668 and ICI 182780 have nearly identical dose response curves for antiuterotrophic activity in immature female rats (Van de Velde et al. 1994). Pharmacokinetic or pharmacodynamic studies with RU 58668 in rats have not been performed. Much of the work concerning RU 58668 has thus far been concerned with its antineoplastic properties (Nique and Van de Velde 1995, Van de Velde et al. 1995). Nevertheless, RU 58668 blocks estrogen stimulated increases in vasoactive intestinal peptide (VIP) mRNA in rat anterior pituitary, and does not stimulate VIP mRNA when administered by itself, indicating that it is a "pure" antiestrogen in this system (Montagne et al. 1995).

Estrogen-dependent behaviors in female rats are a useful model system for studying the neuroendocrine control of mammalian behavior (Beach 1976b). Female sexual behavior is a prime example of an estrogen-dependent behavior which has been extensively studied (Baum et al. 1977, Erskine 1989). Feeding behavior and body weight regulation are two

other estrogen-dependent parameters which are easily measured (Wade and Zucker 1970, Wade 1976). The purpose of this study was to test if RU 58668 blocks these classic estrogen-dependent behaviors. In addition, in vitro nuclear exchange assays were used to assess whether injections of RU 58668 decreased cell nuclear ^{3}H-estradiol binding (Roy and McEwen 1977), and cytosolic ^{3}H-R5020 binding assays were used to determine if RU 58668 blocked estradiol's induction of progestin receptors (Roy et al. 1979, MacLusky and McEwen 1980).

Experimental Design

We used a repeated measures design with three tests, four days apart, to examine if RU 58668 blocked female sexual behavior. The first test (pre) was used to establish a behavioral baseline. On the second test (exp), a random subset of the animals were subcutaneously injected with 20 µg/kg estradiol benzoate and 1 mg/kg RU 58668 at the same time, while the remaining animals only received estradiol benzoate injections. The third behavior test (post) was performed to determine if the antiestrogen's effect on female sexual behavior was reversible.

An in vitro cell nuclear exchange assay was used to determine if injections of RU 58668 reduced cell nuclear ^{3}H-estradiol binding in individual ovariectomized adult female rats (McGinnis et al. 1981). All animals were injected with a saturating dose (Anderson et al. 1972) of unlabeled estradiol 2 hours before the assay (2.7 µg/kg sc), except for the vehicle control and "antiestrogen only" groups, to activate the estrogen receptors. A single 1 mg/kg injection of RU 58668 was given to selected groups 2, 12, 24, or 24/48 hours before the assay. For the RU 24/48 h groups, the animals received 1 mg/kg RU 58668 injections both 24 and 48 hours before the assay.

Cytosolic progestin receptor levels were measured in vitro with single point binding assays (McGinnis et al. 1981) to determine if injections of RU 58668 blocked estradiol's

stimulation of cytosolic ^{3}H-R5020 binding. The animals were given two injections of 20 µg/kg sc estradiol benzoate 48 and 24 hours before the assay to induce the synthesis of cytosolic progestin receptors (Brown et al. 1987). At the same time as the estradiol benzoate injections, the animals were also injected with 1 mg/kg RU 58668, or drug vehicle.

Methods

Drugs

Estradiol benzoate (EB), 17β-estradiol (E_2) and progesterone (P) were purchased from Sigma Chemicals (St. Louis, MO). Estradiol, [2,4,6,7-^{3}H(N)]- and promegestone (R5020), [17α-methyl-^{3}H]- were purchased from DuPont NEN (Wilmington, DE) (Raynaud 1977). RU 58668 (CAS: 151555-47-4) was generously provided by P. Van de Velde at Roussel UCLAF (Romainville, France). A diagram of RU 58668's chemical structure is provided in Appendix A, Figure 51. All injections were given subcutaneously (sc). The steroids were dissolved directly in propylene glycol for the cytosolic progestin receptor assays and the behavioral tests, and polyethylene glycol 200 (PEG-200) for the cell nuclear estrogen receptor assays.

Animal Preparation

Adult male (226-250g) and female (201-225g) Long Evans rats were purchased from Charles River Laboratories (Wilmington, MA). The animals were cared for as described in the General Methods Chapter. Male and female rats were gonadectomized under ethyl ether anesthesia one week prior to testing as previously described (McGinnis et al. 1981, Krey and McGinnis 1990). During gonadectomy, the males received two, 10 mm

Silastic capsules filled with 100% crystalline testosterone to ensure that they would have controlled physiological levels of testosterone throughout the study.

Behavioral Testing

All behavior testing was conducted during the first six hours of the dark period under dim red lighting. Sex behavior testing was conducted in a 25 x 20 x 18 cm glass enclosure. Prior to the experiment the stimulus males were pretested for male sexual behavior with another group of sexually receptive females to confirm that the males were proven copulators. The experimental females were made sexually receptive with two subcutaneous injections of 20 μg/kg estradiol benzoate at -48 hours and -24 hours before the test and then with 2 mg/kg progesterone four hours before the test. The doses of estradiol benzoate and progesterone were chosen to ensure that the females would display high levels of sexual behavior (Morali and Beyer 1979). The antiestrogen dose used in this study was chosen because 1 mg/kg was completely antiuterotrophic in immature rats (Van de Velde et al. 1994).

Body and Food Weight Determination

Body weight and food weight were measured on the day of the antiestrogen-test before the first drug injection (1100 h) and then again 48 hours later with an electronic balance to the nearest 10 mg. Food weight was determined by weighing the food container and its contents (food spillage not accounted for).

Cell Nuclear Estrogen Receptor Assay

Tissue homogenates were prepared from gross brain dissections (pooled preoptic area, hypothalamus, and amygdala) and pituitary tissue with a glass-Teflon homogenizer at

0° C. Cell nuclei were purified with differential centrifugation through sucrose step gradients and then salt extracted to isolate the ligand-bound estrogen receptors. The salt extract containing the receptor complexes was then incubated with 2 nM ^{3}H-estradiol for 5 hours at 24° C and then overnight at 4° C with and without a 100 fold excess of diethylstilbestrol (DES) to correct for non-specific binding. Bound ^{3}H-estradiol was separated from free ^{3}H-estradiol by Sephadex LH-20 chromatography and then measured with scintillation counting. The results were corrected for cell number by normalizing the fmol ^{3}H-estradiol bound to mg DNA (Burton 1956).

Cytosolic Progestin Receptor Assay

Tissue from two rats was pooled for each data point. Tissue homogenates were prepared from grossly dissected brain (pooled hypothalamus-preoptic area) and pituitary tissue using a glass-Teflon homogenizer. These tissues were chosen because they exhibit the highest response of cytosolic ^{3}H-R5020 binding to estradiol benzoate priming (MacLusky and McEwen 1980). The homogenates were then centrifuged at 105,000 g for 45 min at 2° C to pellet cellular organelles. The supernatant (cytosol) was then incubated with 0.5 nM ^{3}H-R5020 at 4°C overnight with and without a 100 fold excess of unlabeled R5020 to correct for non-specific binding. Bound ^{3}H-R5020 was separated from free ^{3}H-R5020 by Sephadex LH-20 chromatography and then measured with scintillation counting. The results (fmol ^{3}H-R5020 bound) were normalized to mg protein (Bradford 1976).

Statistical Analysis

Sexual receptivity was measured by the lordosis quotient (frequency of lordosis postures per ten mounts) and proceptivity was measured by counting the frequency of hopping/darting and ear wiggling events during a five minute period (Beach 1976b).

Results

RU 58668 significantly reduced both the level of sexual receptivity and the level of proceptivity in a reversible manner (repeated measures ANOVA; F (2,42) = 4.307, p = 0.0199; and F (2,42) = 6.886, p = 0.0026; Figure 24). Sexual receptivity in the antiestrogen treated animals as measured by the lordosis quotient was significantly reduced, t (42) = 6.456, $p < 0.0001$, on the second test (exp) and then restored on the third test (post), t (42) = -4.596, $p < 0.0001$ (Figure 24A) . Sexual receptivity did not differ between the three tests in the control animals. Proceptivity was significantly reduced, t (42) = 4.694, $p < 0.0001$, on the second test (exp) (Figure 24B). However, the difference in the level of proceptivity between the second test (exp) and the third test (post) for the antiestrogen treated animals was not significant, t (42) = -1.729, p = 0.0911. Proceptivity did not differ between the three tests in the control animals.

RU 58668 blocked estradiol's effects on body weight and food weight (Figure 25). Vehicle treated animals' body weights gained about 4% while estradiol benzoate treated animals lost about -0.3%. RU 58668 attenuated the EB reduction in body weight over 48 hours (ANOVA, F (2,29) = 15.233, $p < 0.0001$; Figure 25A). RU 58668 also blocked the EB reduction in food weight over 48 hours (ANOVA. F (2,29) = 7.535, $p <$ 0.0023; Figure 25B).

The effects of subcutaneous RU 58668 injections on in vitro cell nuclear ^{3}H-estradiol binding in the brain (ANOVA F (6,21) = 21.20, $p < 0.0001$) and pituitary (ANOVA F (6,21) = 23.06, $p < 0.0001$) of ovariectomized female rats are shown in Figure 26. Injections of RU 58668 were given at several time points (2, 12, 24 or 24/48 hours of exposure) along with unlabeled estradiol (injected 2 h before the assay) to determine if RU 58668 would reduce cell nuclear ^{3}H-estradiol binding in the brain and pituitary differently. In the absence of estradiol (BL group), very low levels of cell nuclear ^{3}H-estradiol binding were found, but estradiol injections given to ovariectomized female

rats 2 h before the assay (E_2 group), significantly increased ($p < 0.0001$ for both brain and pituitary) cell nuclear ^{3}H-estradiol binding relative to the BL group. Two hours after an injection of RU 58668, pituitary cell nuclear ^{3}H-estradiol binding was significantly reduced ($p = 0.0097$) (Figure 26B). However, it should be noted that the 2 hour time point was an *n* of 2, and therefore should be viewed with caution. After 12 hours of RU 58668 treatment, the level of pituitary cell nuclear ^{3}H-estradiol binding was also significantly reduced ($p < 0.0001$). Only when a single injection of RU 58668 was given at least 24 hours before the assay (Figure 26A) was estradiol's stimulation of cell nuclear ^{3}H-estradiol binding significantly reduced ($p = 0.0008$) in the brain. Increasing the duration of RU 58668 exposure by giving two injections of the antiestrogen 24 and 48 hours before the assay was not significantly different from the 24 hour time point. Injections of RU 58668 (24 hours prior to the assay), without a subsequent estradiol injection 2 h before the assay, did not significantly elevate cell nuclear ^{3}H-estradiol binding in either brain or pituitary above drug vehicle levels.

RU 58668 also blocked estradiol's induction of cytosolic ^{3}H-R5020 binding in the brain (ANOVA F (3,20) = 3.45, $p < 0.0362$) and pituitary (ANOVA F (3,20) = 61.20, $p < 0.0001$), shown in Figure 27. In this experiment, the animals received two injections of estradiol benzoate and/or RU 58668 at the same time 48 and 24 hours before the assay. One mg/kg RU 58668 significantly blocked estradiol's stimulation of cytosolic ^{3}H-R5020 binding in the brain (Figure 27A, $p = 0.0338$) and the pituitary (Figure 27B, $p < 0.0001$). When injected alone, RU 58668 did not stimulate cytosolic ^{3}H-R5020 binding in either the brain or the pituitary.

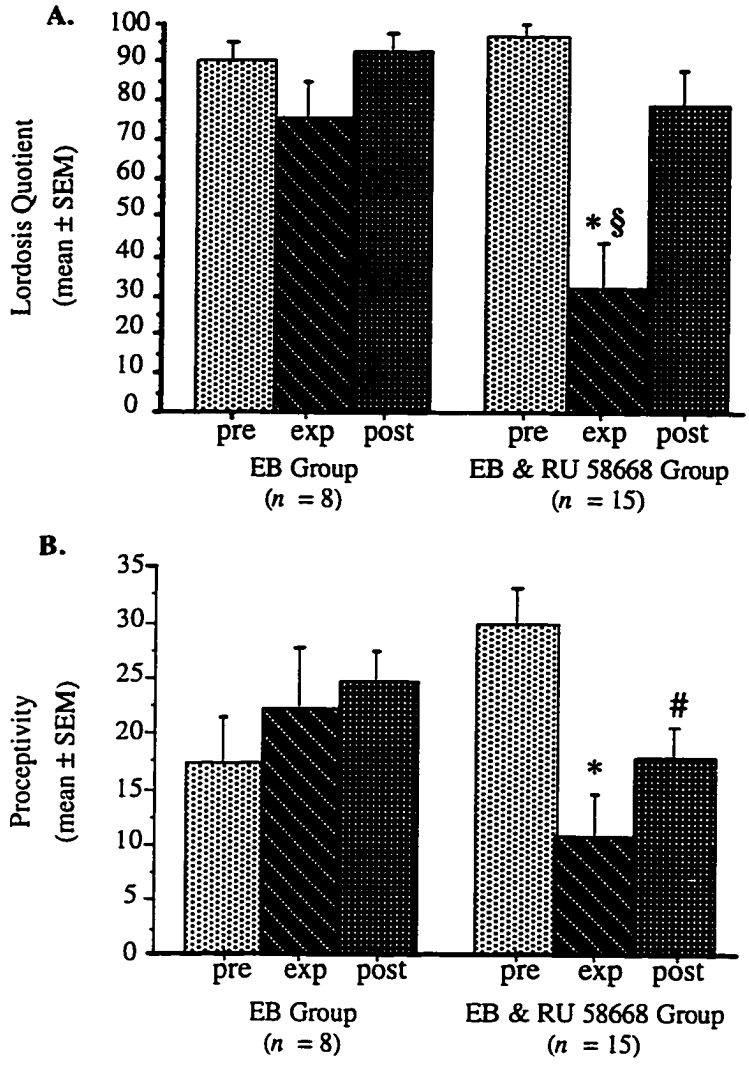


Figure 24. Sexual Receptivity (A) and Proceptivity (B)

Sexual receptivity (A) was measured using the lordosis quotient (percentage of lordosis responses in ten mounts), and proceptivity (B) was measured as the frequency of hopping/darting and ear wiggling during the five minute test. Three consecutive female sex behavior tests were conducted four days apart (pre, exp, and post). For the first test (pre) and the third test (post), all the animals were treated with 20 μg/kg estradiol benzoate (EB) (-48 hours and -24 hours) and 2 mg/kg progesterone (P) (-4 hours). During the second test (exp), the control animals (EB Group) received 20 μg/kg EB (-48 hours and -24 hours) and 2 mg/kg P (-4 hours), while the experimental animals (EB & RU 58668 Group) received 1 mg/kg RU 58668 along with EB and P. (* $p < 0.001$ vs. pre; § $p < 0.001$ vs. post; # $p < 0.01$ vs. pre; Least Squares Means Test)

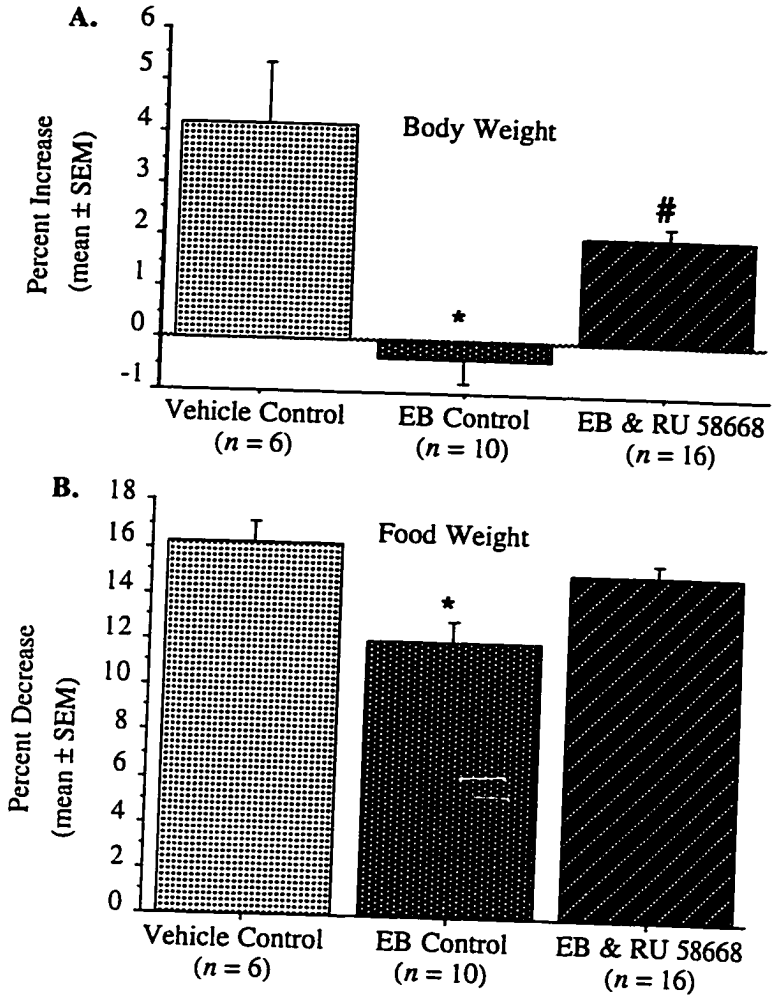


Figure 25. The Effects of RU 58668 on Body Weight (A) and Food Weight (B) in Female Rats

RU 58668 blocked estradiol's suppression of food consumption and body weight gained over a 48 hour period. (* $p < 0.01$ vs. Vehicle Control or 20 mg/kg estradiol benzoate (EB) & 1 mg/kg RU 58668; # $p < 0.01$ vs. Vehicle control; Fisher's PLSD Test)

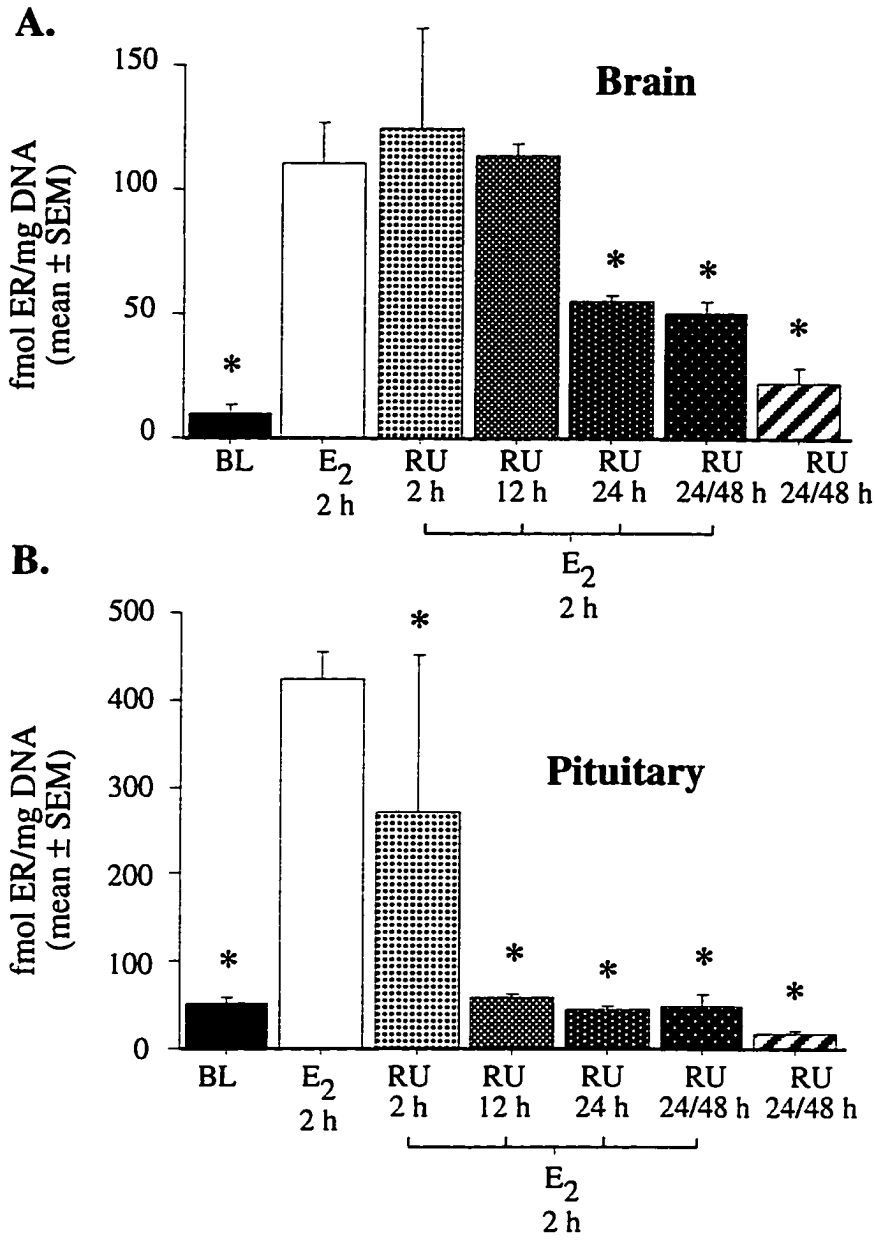


Figure 26. Cell Nuclear Estrogen Receptor Binding

All groups (except for the BL and RU groups) were injected with 2.7 mg/kg estradiol (E_2) 2 hours prior to nuclear exchange assays. The antiestrogen-treated groups were given 1 mg/kg injections of RU 58668 at the times indicated before the assay [BL = vehicle control (PEG-200)(n = 4); E_2 = estradiol alone 2 hours before the assay (n = 4); E_2 & RU 2 h = estradiol and RU 58668 both given 2 hours before the assay (n = 2); E_2 & RU 12 h = estradiol given 2 hours before the assay and RU 58668 given 12 hours before the assay (n = 6); E_2 & RU 24 h = estradiol given 2 hours before the assay and RU 58668 given 24 hours before the assay (n = 4); E_2 & RU 24/48 h = estradiol given 2 hours before the assay and RU 58668 given 24 and 48 hours before the assay (n = 4); RU = RU 58668 given 24 and 48 hours before the assay (n = 4)]. RU 58668 significantly reduced cell nuclear ^{3}H-estradiol binding in the brain (pooled preoptic area, hypothalamus, and amygdala) 24 hours after it was injected. In contrast, pituitary cell nuclear ^{3}H-estradiol binding was significantly reduced 2 hours after RU 58668 was injected. Asterisks indicate $p < 0.05$ vs. E_2, Fisher's PLSD test.

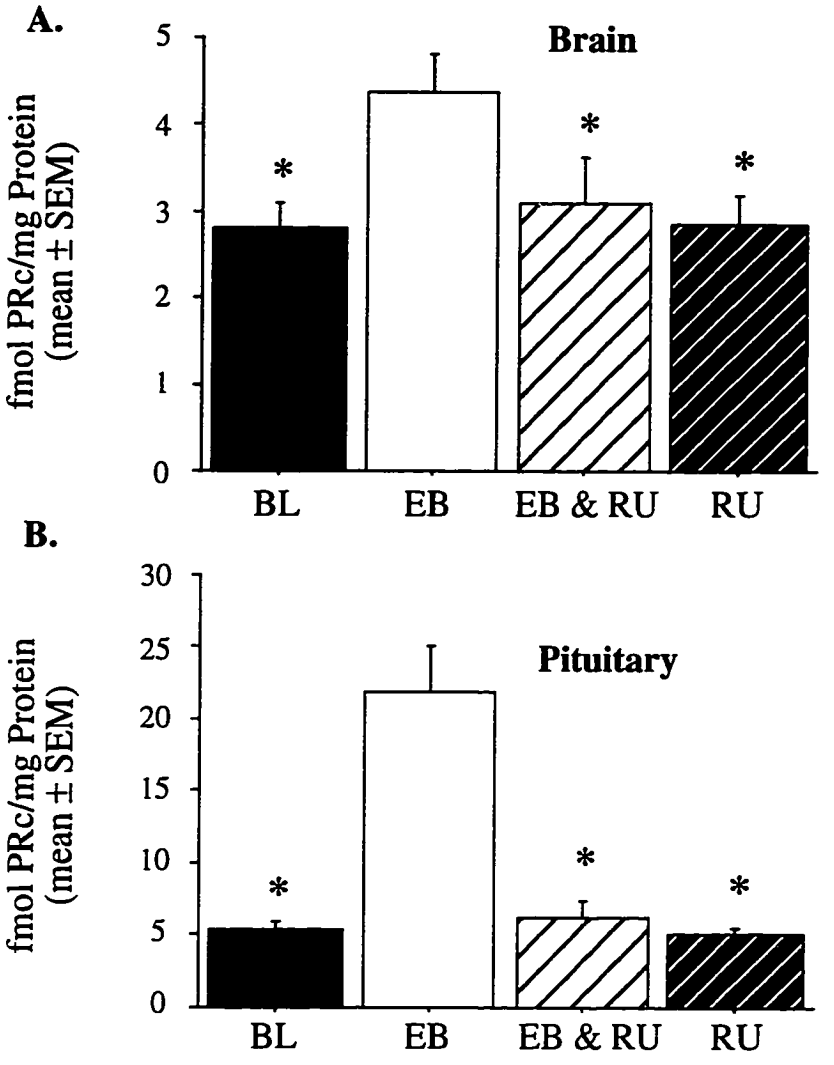


Figure 27. Cytosolic ^{3}H-R5020 Binding

Cytosolic ^{3}H-R5020 binding was measured in vitro on tissue from ovariectomized adult female rats. Two injections were given 48 and 24 hours before the assay (BL = propylene glycol, EB = 20 mg/kg estradiol benzoate, EB & RU = 20 mg/kg estradiol benzoate and 1 mg/kg RU 58668, RU = 5 mg/kg RU 58668). There were six data points in each group. RU 58668 significantly blocked estrogen's induction of cytosolic ^{3}H-R5020 binding in the brain (pooled preoptic area and hypothalamus) and pituitary. When injected by itself, RU 58668 did not stimulate cytosolic ^{3}H-R5020 binding in either brain or pituitary. Asterisks indicate $p < 0.05$ vs. E_2, Fisher's PLSD test.

Discussion

The results show that RU 58668 is an effective antiestrogen on measures of female sexual behavior and feeding behavior. RU 58668 significantly decreased lordosis behavior in the antiestrogen-treated rats. Furthermore, the effect was reversible because after allowing the drug to clear for four days, the antiestrogen-treated rats' lordosis quotient levels in the third test (post) were restored to similar levels found in the first test (pre). The results with RU 58668 are in agreement with similar studies that have used other antiestrogens to block lordosis behavior (Arai and Gorski 1968, Baum and Vreeburg 1976, Morin et al. 1976, Roy and Wade 1977, Blaustein et al. 1979). However, the differences between RU 58668's and ICI 182780's effects on female sexual behavior are striking. The reduction of female sexual behavior was observable with as little as 48 hours of RU 58668 exposure. In contrast, only after chronic exposure for 24 days did ICI 182780's suppression of female sexual behavior become apparent, even though a similar experimental paradigm was employed in rats (Wade et al. 1993a). This reduction in female rat sexual behavior following chronic treatment with ICI 182780 might be due to blocking peripheral sites necessary for female sexual behavior (Wade et al. 1993a). It is interesting that unlike rats, hamster female sexual behavior can be reduced by two days of treatment with ICI 182780. However, this might be due to a species difference in the estrogenic regulation of female sexual behavior between rats and hamsters (Siegel et al. 1979, Ahdieh and Wade 1982, Wade et al. 1993b).

Proceptivity was significantly reduced by the antiestrogen treatment, but the magnitude of the changes in proceptivity caused by RU 58668 were not as marked as the reduction in sexual receptivity. Moreover, the level of proceptivity on the third test (post) was not significantly increased relative to the level of proceptivity on the second test (exp) in the RU 58668 treated rats. It is difficult to determine whether the effects on proceptivity

were reversible due to the variability shown in the proceptive behaviors between the test sessions.

Estrogen treatment given to ovariectomized rats results in a decrease in the amount of body weight gained and the amount of food consumed (Clark and Tarttelin 1982, Devenport and Torres 1984). RU 58668 is the only antiestrogen which does not have estrogenic effects on body weight and feeding behavior in female rats. Type I antiestrogens such as tamoxifen, CI-628, and MER-25 all function as estrogens when assayed on feeding behavior or body weight (Roy et al. 1977, Wade and Blaustein 1978, Wade and Heller 1993). ICI 182780, a type II antiestrogen, differs from other previously studied antiestrogens in that it is not estrogenic on either body weight or food intake (Wade et al. 1993a). However, ICI 182780 also does not block estrogen's suppression of body weight or food intake in female rats.

The central nervous system's role in regulating female sexual behavior and feeding behavior is well known (Dudley 1982). Studies on ICI 182780 (which does not reach the central nervous system) show that peripheral estrogen receptor action can affect both female sexual behavior and feeding behavior to some extent (Wade et al. 1993a). However, the differences between RU 58668's effects on these behaviors and ICI 182780's effects on these behaviors indicates that brain estrogen receptor action plays a pivotal role in mediating female sexual behavior and feeding behavior. Since RU 58668 effectively blocked these estrogen-dependent brain-mediated behaviors, it is likely that it can cross the blood-brain barrier and block brain estrogen receptors.

The binding of estradiol to cell nuclear estrogen receptors results in a complex sequence of events (known as receptor activation or transformation) culminating in the either the stimulation or repression of target-gene transcription (Gorski et al. 1993). The biochemical differences between unbound and ligand-bound steroid receptors can be harnessed in nuclear exchange assays to provide a measure of ligand-activated nuclear receptors (Anderson et al. 1972, Roy and McEwen 1977, Roy 1978, Roy and McEwen

1979, Krey et al. 1980, Sica et al. 1981, Kirchhoff et al. 1983, Leavitt and Okulicz 1985, Lustig et al. 1989). In these assays, the endogenous bound ligand is exchanged for exogenous ^{3}H-estradiol in vitro, to provide a measure of how much ligand-bound receptor is present in the cell nucleus (Roy and McEwen 1977, McGinnis et al. 1981). We found that RU 58668 blocks the estradiol-stimulated increase in cell nuclear ^{3}H-estradiol binding. RU 58668 is distinct from tamoxifen (Jordan et al. 1977, Kirchhoff et al. 1983) and CI-628 (Capony and Rochefort 1975, Roy 1978, Roy and McEwen 1979) because RU 58668 does not increase cell nuclear ^{3}H-estradiol binding when it is injected in the absence of estradiol.

It took up to 24 hours after a single injection of RU 58668 to block estradiol-stimulated cell nuclear ^{3}H-estradiol binding in brain tissue. This was much longer that it took to observe the same response in the pituitary, which is outside the blood-brain barrier. It is possible that the difference in molecular weight between estradiol and RU 58668 (272.4 and 658.76 respectively) and/or the constituents of RU 58668's 11β side chain played a role in the length of time it took to enter the brain (Pardridge 1994). However, the fact that RU 58668 could even reach the brain was intriguing because another steroidal antiestrogen, ICI 182780, which has a large 7α side chain, reportedly does not enter the brain (Wade et al. 1993a).

There are a number of different explanations for how RU 58668 could have decreased cell nuclear ^{3}H-estradiol binding, but I would like to stress that the focus of this work was not to determine the mechanism of action of RU 58668 in the brain. Currently, the mechanism of RU 58668's action is poorly understood, and much of what is known has been inferred from studies on other pure antiestrogens, such as ICI 182780 and its congeners. First, RU 58668 could have prevented the nuclear transformation of estrogen receptors, and thus, lowered the level of cell nuclear ^{3}H-estradiol binding. Second, RU 58668 might have been bound to the estrogen receptors so tightly that it could not exchange with the ^{3}H-estradiol in the assay. However, the relative binding affinity of

RU 58668, with respect to labeled estradiol, for mouse uterine estrogen receptors in vitro is 56% (Van de Velde et al. 1994). Therefore, ^{3}H-estradiol would be expected to exchange with bound RU 58668. Third, RU 58668 might induce estrogen receptor protein degradation, and this could also have lowered the level of cell nuclear ^{3}H-estradiol binding. This possibility is likely because work in cell culture has shown that RU 58668 reduces the level of estrogen receptor protein (Jin et al. 1995).

The levels of hypothalamic and pituitary progestin receptors are known to be increased by estradiol treatment (Roy et al. 1979, MacLusky and McEwen 1980, Etgen 1981, Clark et al. 1982a, Etgen and Shamamian 1986, Lauber et al. 1991c). Thus, cytosolic ^{3}H-R5020 binding can be used as an in vitro assay of endogenous estrogen receptor action. The antiestrogens, CI-628 and tamoxifen, both reduce estrogen's induction of cytosolic progestin receptors in the brain and pituitary (Roy et al. 1979, Etgen and Shamamian 1986). However, they are also estrogenic, in that they induce cytosolic progestin receptors in the absence of estradiol. The results show that RU 58668 blocks estradiol-stimulated increases in cytosolic ^{3}H-R5020 binding in brain, pituitary, and uterus without having estrogenic effects of its own.

In conclusion, RU 58668 significantly blocked estrogen's facilitation of female sexual behavior and blocked estrogen's suppression of feeding behavior and body weight. Principally, RU 58668 is a "pure" antiestrogen which can block brain mediated behaviors. Moreover, subcutaneous injections of the steroidal antiestrogen, RU 58668, decreased the level of in vitro cell nuclear ^{3}H-estradiol binding in the female rat brain and pituitary. RU 58668 does get into the central nervous system, but more slowly than estradiol. Furthermore, RU 58668 blocked estradiol-stimulated increases in cytosolic ^{3}H-R5020 binding. RU 58668 is not estrogenic in either of these two assays when injected in the absence of estradiol. These findings indicate that RU 58668 is an effective estrogen receptor antagonist for neuroendocrine studies.

Part 4: Effects of the Antiestrogen RU 58668 on Hormone-Dependent Sociosexual Behaviors

Introduction

The purpose of this experiment was to test if the antiestrogen RU 58668 would inhibit the restoration of hormone-dependent sociosexual behaviors in testosterone-treated gonadectomized rats. In spite the fact that the pure antiestrogen ICI 182780 did not block male copulatory behavior, my hypothesis was that the restoration of male copulatory behavior was mediated, in part, by the action of cell nuclear estrogen receptors, and that this process could be blocked with a pure antiestrogen. RU 58668 appeared to be a good candidate for a pure antiestrogen, based on my results with this compound blocking several estrogen-dependent measures in female rats. One of the most substantial findings from the experiments with female rats, described in the previous section, was that RU 58668 blocked brain cell nuclear estrogen receptor binding. This indicated that it would be possible to determine if this antiestrogen blocked brain cell nuclear estrogen receptors in male rats. The reason the ability to measure brain estrogen receptor action was important was because it would allow for the results of the behavior tests to be interpreted considering whether brain estrogen receptors were really blocked.

Experimental Design

This experiment was based on a restoration paradigm for male copulatory behavior, and therefore, was conceptually similar to the previous studies in male rats with fadrozole and ICI 182780. However, a major difference in this study was that the drug was not delivered by the intracerebroventricular (ICV) route, but by daily subcutaneous injections. The reason ICV delivery was not necessary was because RU 58668 could cross the blood-

brain barrier. Attempts were made at using subcutaneous miniature osmotic pumps to deliver RU 58668 systemically, however technical problems prompted me to use daily injections. The problem was that RU 58668 is so hydrophobic that it aggregated in the saline solution when the pumps were primed before implant surgery. Later, I found that attaching a cannula to the pump, extending away from the saline, prevented the pump from being clogged. However, since the experiment had already begun, I continued using daily injections to deliver the RU 58668. Another difference in this study relative to the previous experiments was that a scent marking/ultrasonic vocalization test was included with the tests for partner preference and male copulatory behavior. Both scent marking and 50 kHz ultrasonic vocalizations, in response to estrus bedding, have been previously shown to be testosterone-dependent in male rats. Testing for these other hormone-dependent behaviors in the same study provided for a more integrative approach by covering a wider range of behaviors.

Methods

The methods used for animal preparation, behavioral testing, data collection and analysis, and cell nuclear estrogen receptor assays were the same as described in the General Methods Chapter. The dose for RU 58668 was chosen based upon a report that 3 mg/kg resulted in maximum antiuterotrophic activity in immature female mice (Van de Velde et al. 1994). This dose was three times higher than the dose used in part 3 of this chapter, where RU 58668 was found to reduce several estrogen-dependent measures in female rats. In addition, 15 mg/kg sc RU 58668 was also tested to determine if there could be a further reduction in the behaviors or on cell nuclear estrogen receptor binding.

Results

The effects of 3 and 15 mg/kg sc RU 58668 on the restoration of ejaculatory behavior are displayed in Figure 28. RU 58668 did not have any effect on testosterone's restoration of ejaculatory behavior (test #1 $\chi^2(2, N = 34) = 0.404$, $p = 0.8172$; test #2 $\chi^2(2, N = 34) = 1.262$, $p = 0.5321$). The data for the percentage of males mounting and the percentage of males intromitting were similar, so they were not shown. Analysis of individual measures of male copulatory behavior did not reveal any significant trends over both tests that were dose-dependent (Appendix A, Table 19). Therefore, RU 58668 did not impair the restoration of male copulatory behavior.

RU 58668 did not impair the restoration of partner preference for either the time spent with the nonreceptive female subtracted from the time spent with the receptive female (group x test interaction, repeated measures ANOVA $F(4,62) = 0.93$, $p = 0.4511$, Figure 29), or for the time spent in the empty chamber (group x test interaction, repeated measures ANOVA $F(4,62) = 2.180$, $p = 0.0816$, Appendix A, Figure 54). On the other hand, daily injections of 3 mg/kg sc RU 58668 reduced testosterone's restoration of boundary crossing frequency (repeated measures contrast on the test x group interaction, ANOVA $F(4,62) = 3.56$, $p = 0.0111$ vs. T, Appendix A, Figure 55). However, this effect was not evident in the "T & 15 mg/kg sc RU 58668" group.

The effects of antiestrogen treatment on testosterone's restoration of 50 kHz vocalizations and scent marking are depicted for each individual animal in Appendix A, Figure 56 and Figure 57, respectively. These figures illustrate the large amount of interanimal and intertest variability in this data. Therefore, the statistical analysis was performed on the paired difference scores between tests as described in the General Methods Chapter, rather than on the raw data. Treatment with RU 58668 did not affect the restoration of 50 kHz vocalizations during the first week of testosterone treatment (ANOVA $F(2, 24) = 2.36$, $p = 0.1160$, Figure 30A). However, both doses of the antiestrogen were

significantly different from the testosterone group on the interval between test #1 and test #2 (ANOVA F (2, 24) = 7.31, p = 0.0033, Figure 30B). With respect to scent marking, treatment with RU 58668 did not have any significant effect at either test interval (test #1 - postcastration test, ANOVA F (2, 24) = 3.12, p = 0.0623, Figure 31A; test #2 - test #1, ANOVA F (2, 24) = 0.426, p = 0.6624, Figure 31B). However, during the first test interval, the large amount of interanimal variability might have masked the effect of the antiestrogen, because both doses of RU 58668 do appear to reduce the restoration of scent marking relative to testosterone (see Figure 31A).

Nuclear estrogen receptor assays were performed to determine if RU 58668 blocked brain estrogen receptors. These data are shown in Figure 32 (ANOVA F (4, 24) = 12.58, p = 0.0001). Gonadectomized males have significantly lower levels of cell nuclear estrogen receptor binding than gonadectomized males treated with testosterone (p = 0.0001). Both 3 mg/kg (p = 0.0015) and 15 mg/kg (p = 0.0002) RU 58668 significantly reduced the level of cell nuclear estrogen receptor binding in testosterone-treated gonadectomized males relative to gonadectomized males treated with testosterone (positive controls). However, neither dose of the antiestrogen was significantly different from the gonadectomized males without testosterone.

In addition, a separate group of testosterone-treated gonadectomized males received two injections (48 and 24 hours before the assay) of 30 mg/kg sc RU 58668. The results from the nuclear androgen receptor assay performed on these animals is shown in Figure 33. Just like in the nuclear estrogen receptor assay, gonadectomized males had significantly lower levels of cell nuclear androgen receptor binding ($p < 0.05$) than gonadectomized males treated with testosterone. However, RU 58668 did not affect the level of brain cell nuclear androgen receptor binding relative to the testosterone-treated gonadectomized males ($p > 0.05$).

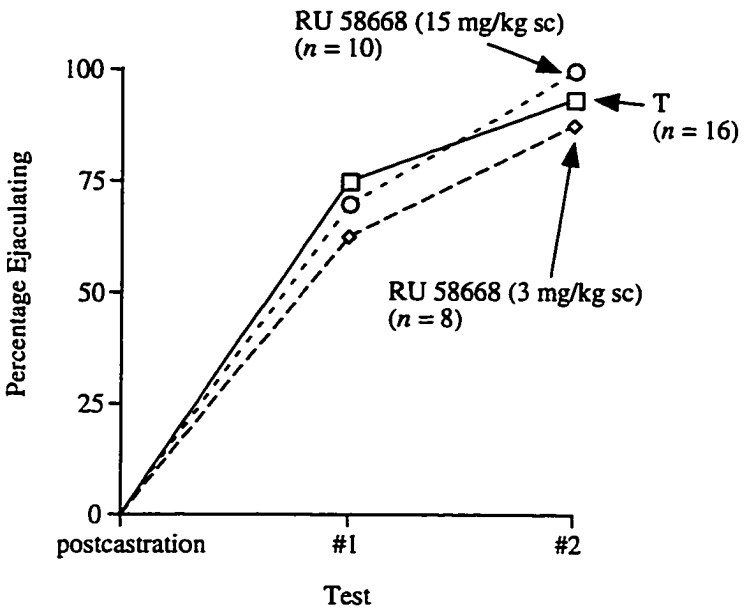


Figure 28. Effect of RU 58668 on the Restoration of Ejaculatory Behavior

Test #1 was 7 days after implant surgery and test #2 was 13 days after implant surgery. Males receiving daily injections of RU 58668 were also implanted with two, 10 mm Silastic capsules filled with testosterone. Neither dose of RU 58668 significantly affected the percentage of males ejaculating on either test #1 or test #2 relative to the testosterone group (Fisher's Exact tests).

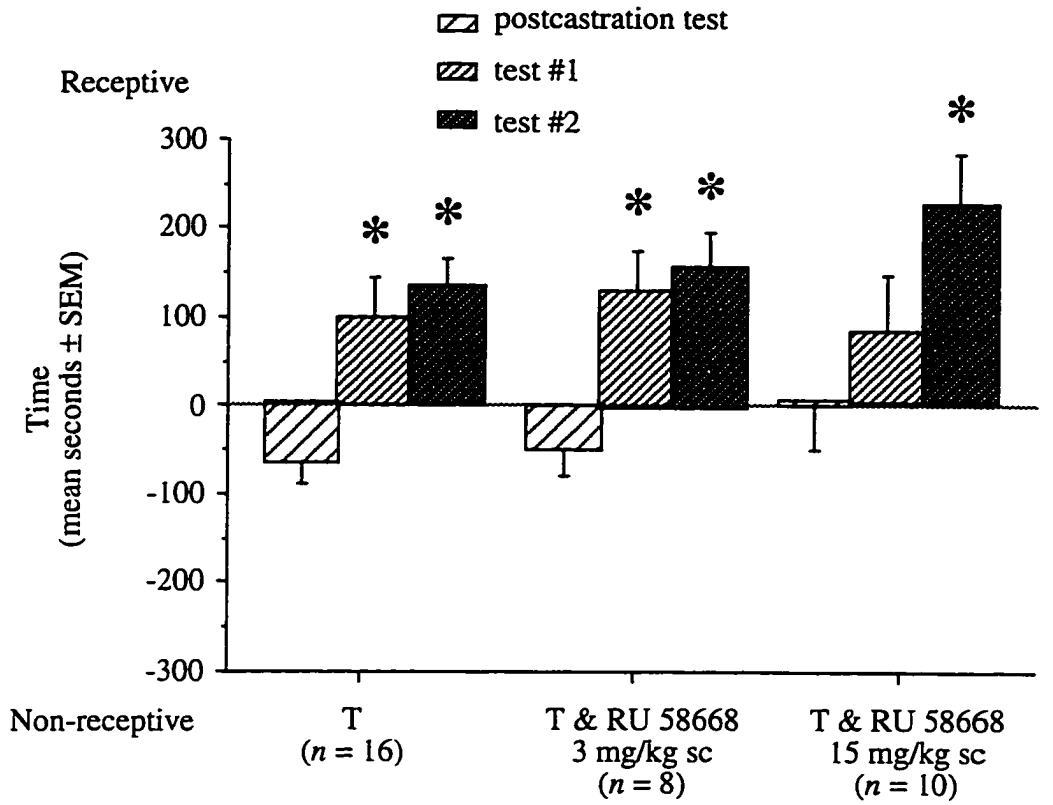


Figure 29. Effect of RU 58668 on the Restoration of Partner Preference

Partner preference test (test #1, 7 days postcastration; test #2, 13 days postcastration) T = testosterone-treated gonadectomized males. The y-axis depicts time spent with the non-receptive female subtracted from time spent with the receptive female. Positive values indicate that more time was spent with the receptive female and vice versa.

* $p < 0.05$, within group comparison vs. postcastration test, Least Square Mean Test

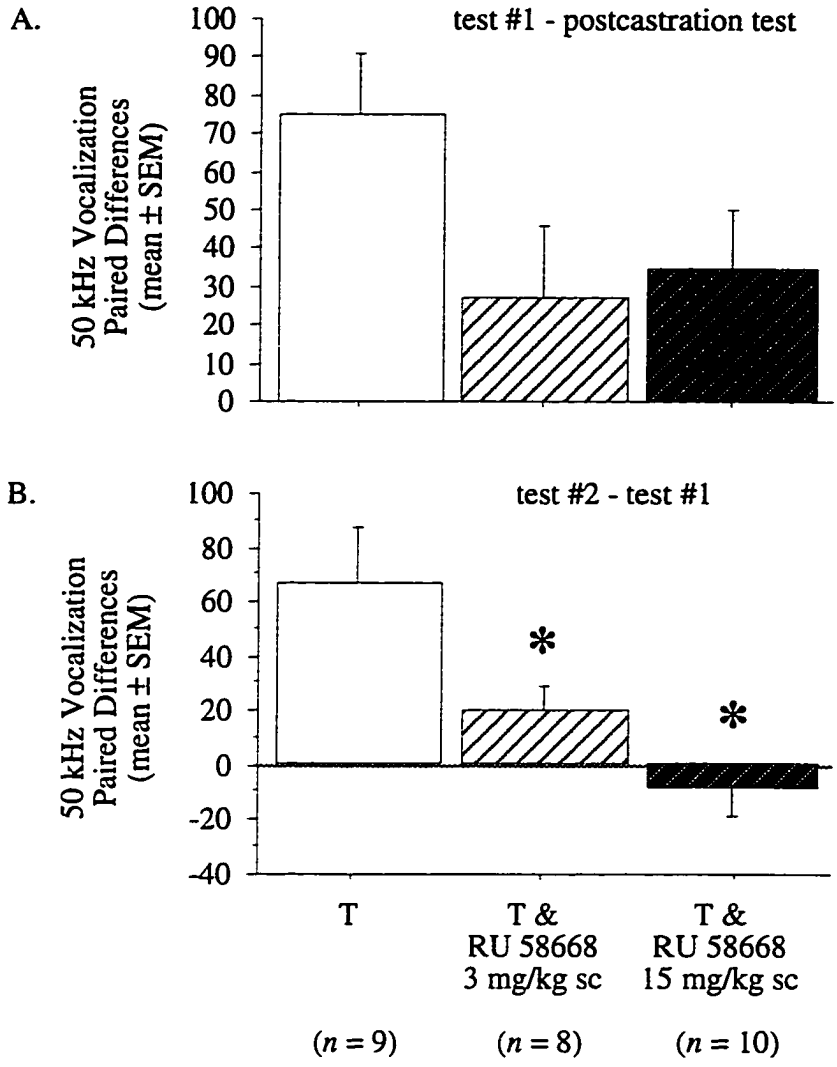


Figure 30. Effect of RU 58668 on the Restoration of 50 kHz Vocalizations

Panel A displays the paired difference data for the first week's interval (test #1 - postcastration test), and panel B displays the paired difference data for the second week's interval (test #2 - test #1).
* $p < 0.05$, Fisher's PLSD test

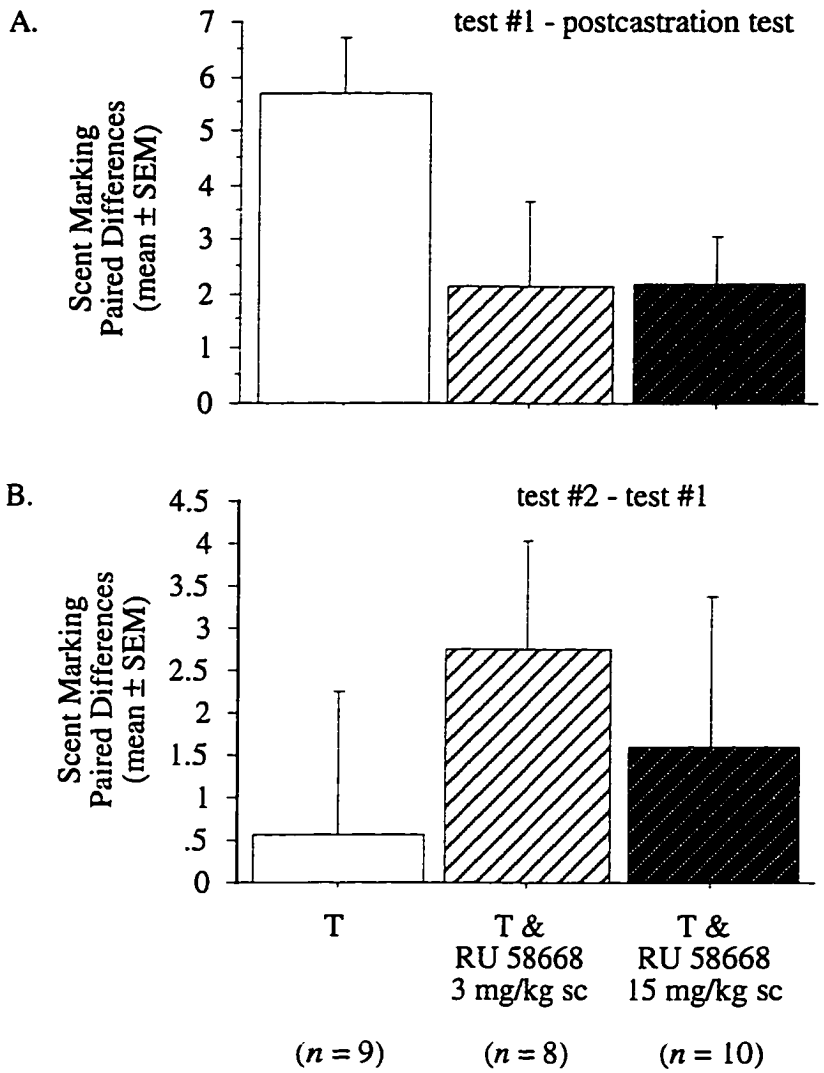


Figure 31. Effect of RU 58668 on the Restoration of Scent Marking

Panel A displays the paired difference data for the first week's interval (test #1 - postcastration test), and panel B displays the paired difference data for the second week's interval (test #2 - test #1).
* $p < 0.05$, Fisher's PLSD test

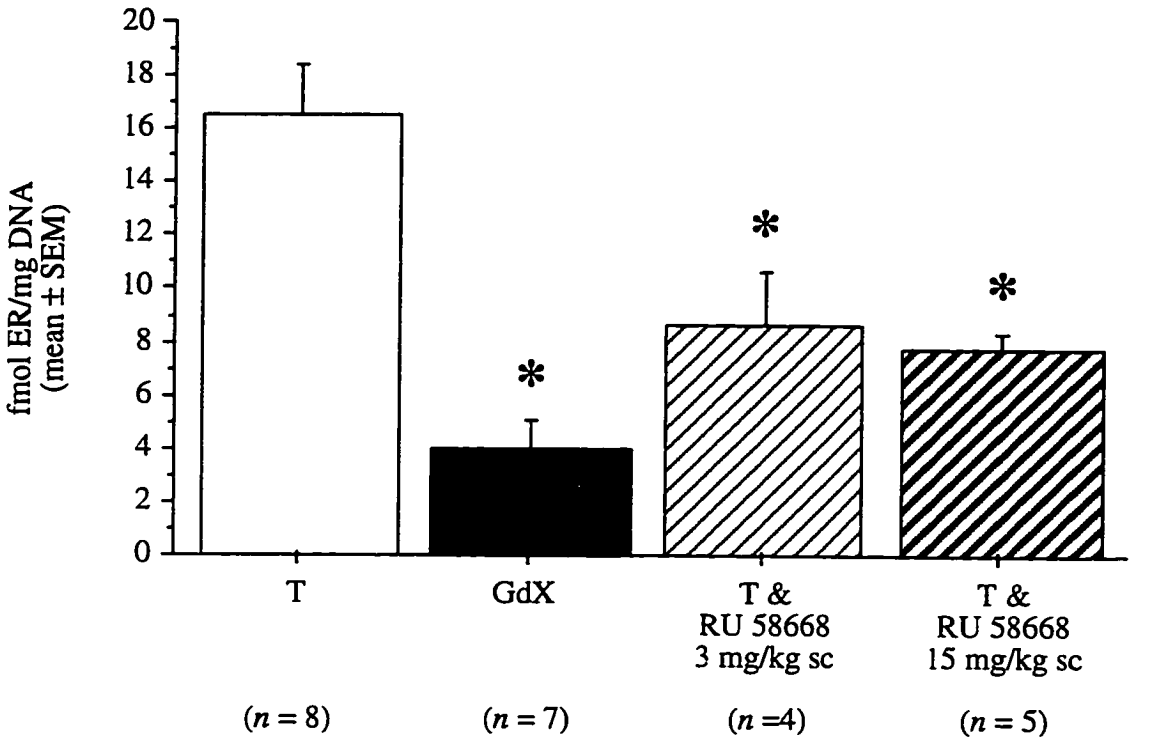


Figure 32. Effect of RU 58668 on Cell Nuclear Estrogen Receptor Binding

Nuclear estrogen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala)(T = gonadectomized male implanted with testosterone capsules; GdX = gonadectomized male).
* $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

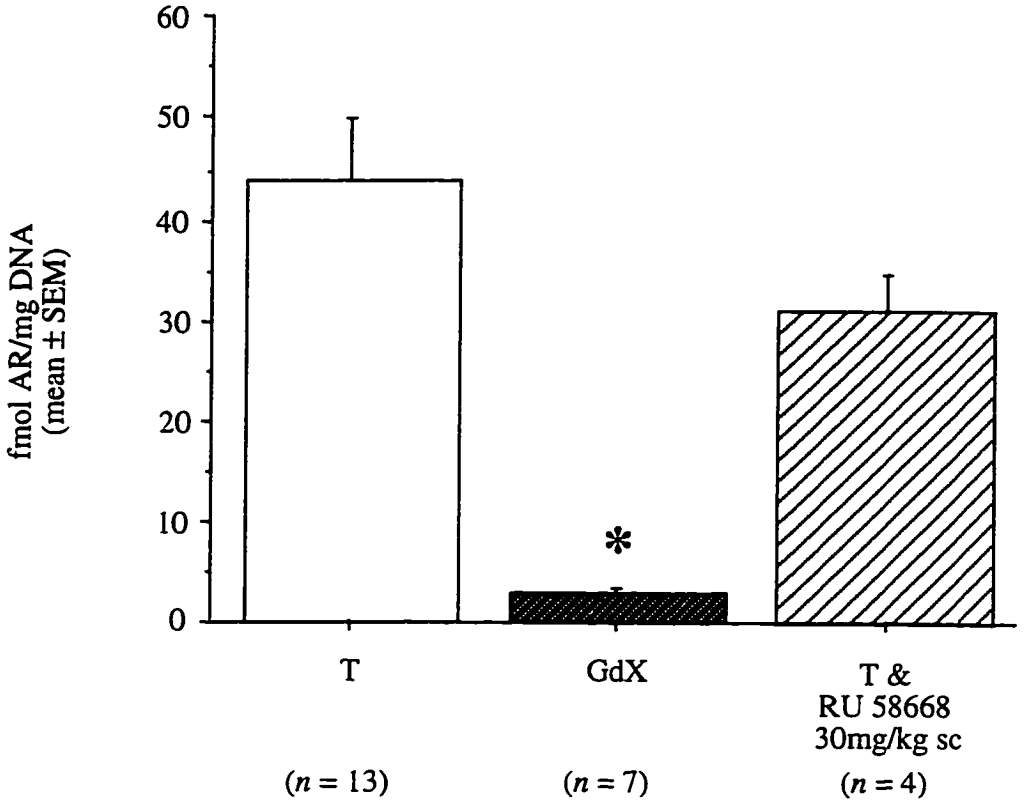


Figure 33. Effect of RU 58668 on Cell Nuclear Androgen Receptor Binding

Nuclear androgen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala)(T = gonadectomized male implanted with testosterone capsules; GdX = gonadectomized male).
* $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

Discussion

The purpose of this study was to determine the role of estrogen receptor action in the restoration of several hormone-dependent sociosexual behaviors in adult male rats. The results show that treatment with the antiestrogen RU 58668 does not significantly block testosterone's restoration of male copulatory behavior, partner preference, or scent marking. However, there was a trend indicating that RU 58668 might impair the restoration of scent marking. The restoration of 50 kHz vocalizations was blocked during the second week of hormone treatment, although it is interesting to note that RU 58668 did not affect the restoration of 50 kHz vocalizations during the first week of hormone treatment.

Little is known about the estrogen-dependence of the other behaviors studied here, and I am not aware of any work with studying the effects of antiestrogens on these behaviors in male rats. Partner preference does not appear to be an estrogen-dependent behavior, because treatment with the aromatase inhibitor fadrozole does not block the testosterone's restoration, and treatment with a physiological dose of estradiol alone does not restore partner preference. Therefore, it is not surprising that RU 58668 did not affect the restoration of partner preference.

On the other hand, my results for 50 kHz vocalizations and scent marking are surprising. Matochik and Barfield (1991), showed that treatment with estradiol alone was sufficient to restore scent marking, but not 50 kHz vocalizations. My results showed that antiestrogen treatment blocked the restoration of 50 kHz vocalizations. The physiological significance of the antiestrogen only affecting the restoration of 50 kHz vocalizations during the second week of hormone treatment is unclear, but it might indicate that multiple processes are involved in the restoration of this behavior.

The effect of RU 58668 on scent marking was not statistically significant with the ANOVA used in the results section (see Figure 31A). However, there was a strong trend indicating that 15 mg/kg dose, but not 3 mg/kg dose of RU 58668 was sufficient to inhibit the restoration of scent marking. When the 15 mg/kg RU 58668 group was compared with the T group using a *t*-test, instead of an ANOVA with all three groups, the scent marking result was statistically significant ($t(17) = -2.575$, $p = 0.0195$). However, the 3 mg/kg RU 58668 group was not statistically different ($t(15) = -1.395$, $p = 0.0721$) from the T group. Therefore, the 3 mg/kg RU 58668 group could have masked the effect of the 15 mg/kg group in the ANOVA. Since Matochik's and Barfield's (1991) data showed that estradiol alone was sufficient to restore scent marking, it is likely that scent marking is an estrogen-dependent behavior. Therefore, the argument is being made that antiestrogen-treatment can impair testosterone's restoration of scent marking.

As mentioned in Chapter 4, estradiol plays a central role in the restoration of male copulatory behavior, and it is commonly thought that estradiol acts in the brain by binding to classical nuclear estrogen receptors (Krey et al. 1980). However, the belief that estradiol acts in male rats to restore male copulatory behavior through the activation of nuclear estrogen receptors is not well supported by the data, and there is little consensus as to whether antiestrogens really block the restoration male copulatory behavior (reviewed in Perez Palacios et al. 1975, Landau 1986). To date, for every study concluding that different antiestrogens blocked the restoration of male copulatory behavior, there are other studies reporting opposite results. For example, Luttge (1975) reported that CI-628 significantly reduced male copulatory behavior, but Yahr and Gerling (1978) reported just the opposite. This was also the case with tamoxifen, where Beyer et al. (1976b) reported that tamoxifen significantly reduced male copulatory behavior, but McGinnis et al. (1990) was not able to replicate this finding. Besides these inconsistent results being reported, some antiestrogens such as MER-25 actually facilitates the restoration of male copulatory

behavior in conjunction with testosterone (Luttge 1975, Baum and Vreeburg 1976, Beyer et al. 1976b).

One reason cited for these inconsistent effects on male copulatory behavior is the fact that these antiestrogens (MER-25, CI-628, and tamoxifen) are all potent estrogens by themselves, depending on the tissue they are tested in (Landau 1986, McDonnell et al. 1995, Jensen 1996). Therefore, it is possible that these antiestrogens were acting as estrogenic compounds in the adult male rat brain. However, it is interesting that these same antiestrogens have been shown to block the effects of estradiol on brain sexual differentiation in neonatal male rats (Sodersten 1978, Vega Matuszczyk and Larsson 1995), and that these antiestrogens also block female copulatory behavior (Morin et al. 1976, Roy and Wade 1977, Wade and Blaustein 1978, Meisel et al. 1987). RU 58668 is different from these nonsteroidal antiestrogens in that it is not a partial agonist (Van de Velde et al. 1994).

In addition to the behavioral results in this study, RU 58668 also reduced the level of brain nuclear estrogen receptor binding to the level found in gonadectomized males, without affecting brain androgen receptor binding. The fact that RU 58668 blocked brain estrogen receptor binding, but not male copulatory behavior is one of the most important aspects of this study, because it shows that male copulatory behavior can be restored by testosterone-treatment in the absence of estrogen receptor action. This is also one of the most notable differences between the current study and the previous studies, because this is the first time that the effects of an antiestrogen have been correlated with the results from a biochemical assay indicating whether or not the antiestrogen was effective. The results of the nuclear estrogen receptor assay also indicate that 3 mg/kg sc RU 58668 is sufficient to bring the level of nuclear estrogen receptor binding down to the level found in gonadectomized male rats, because a 5x greater does did not result in a further repression of brain nuclear estrogen receptor binding. Therefore, it is unlikely that the dose of RU 58668 was not sufficient or that the drug did not reach the brain. In conclusion, the

results of this study indicate that estrogen does not act through classical estrogen receptors during the restoration of male copulatory behavior in rats.

Chapter 6 - Effects of Blocking Both Androgen and/or Estrogen Receptors on the Restoration of Hormone-Dependent Sociosexual Behaviors

In contrast to the effects of antiestrogens in male rats, copulatory behavior can be blocked by treatment with antiandrogens (see Table 12 for references). Three antiandrogens have been used in the study of male copulatory behavior in rats: cyproterone acetate, flutamide, and hydroxyflutamide. However, it is difficult to directly compare the results from studies using these compounds, because only one study (McGinnis and Mirth 1986) actually measured the effects of the antiandrogen on brain androgen receptors. Furthermore, cyproterone acetate is a partial agonist with enough androgenic activity to stimulate male copulatory behavior by itself (Bloch and Davidson 1971). Therefore, the efficacy of these compounds in blocking the restoration of male copulatory behavior cannot be compared, because in most cases it is not known how much the antiandrogens inhibited brain cell nuclear androgen receptor action.

A comparison of the studies listed in Table 12 reveals that the effectiveness of antiandrogen treatment depends on the behavioral paradigm used. For example, no studies have shown that the maintenance of male copulatory behavior can be inhibited by antiandrogen treatment, while the restoration of male copulatory behavior can be inhibited by antiandrogen treatment. This indicates that the maintenance and the restoration of male copulatory behavior are mediated by distinct mechanisms. The idea that multiple mechanisms could be involved in mediating male copulatory behavior is not surprising since two different hormones (testosterone and estradiol) are also known to regulate this behavior. However, because the maintenance of male copulatory behavior could not be

Table 12. Survey of the Effects of Antiandrogens on Male Copulatory Behavior in Rats

Antiandrogen	Behavior Paradigm	Drug Delivery	Behavior Blocked?	Study
cyproterone acetate	maintenance	A	no	(Whalen and Edwards 1969)
cyproterone acetate	maintenance	A	no	(Beach and Westbrook 1968b)
cyproterone acetate	maintenance	A	no	(Zucker 1966)
cyproterone acetate	restoration	A	yes	(Luttge et al. 1975)
flutamide	restoration	A	yes	(Sodersten et al. 1975)
flutamide	maintenance	A	no	(Sodersten et al. 1975)
flutamide	restoration	A	yes	(Gray 1977)
flutamide	restoration	A	yes	(Gladue and Clemens 1980)
hydroxyflutamide	maintenance	A	no	(McGinnis and Mirth 1986)
hydroxyflutamide	restoration	A	yes	(McGinnis and Mirth 1986)
hydroxyflutamide	restoration	B	yes	(McGinnis et al. 1996)

Note: The criteria used to compare the effectiveness of the antiandrogens used in these studies was based on whether or not the antiandrogen blocked the effect of testosterone on ejaculatory behavior.
Abbreviations: A = daily peripheral injections, B = intracranial implants

inhibited with antiandrogen treatment in previous studies, only a restoration paradigm was used in my work.

It is also interesting to note that hydroxyflutamide (SCH 16423) is a naturally occurring metabolite of flutamide (SCH 12321) produced by flutamide's hydroxylation in the liver. In fact, the majority of flutamide is converted to hydroxyflutamide, which is responsible for flutamide's antiandrogenic activity (reviewed in: Brogden and Clissold 1989). A diagram of hydroxyflutamide's chemical structure is provided in Appendix A, Figure 51. In cases where flutamide (Sodersten et al. 1975, Gladue and Clemens 1980) and hydroxyflutamide (McGinnis and Mirth 1986) have been administered peripherally, similar results on male copulatory behavior in rats have been found. Intracranial implant studies with flutamide have not been conducted, but since the drug is inactive until it passes through the liver, it would be unlikely that intracranial implants of flutamide would inhibit the restoration of male copulatory behavior. An intracranial implant study with hydroxyflutamide has shown inhibition of androgen receptors in the medial preoptic area or the ventromedial hypothalamus can inhibit the restoration of male copulatory behavior

(McGinnis et al. 1996). Therefore, brain androgen receptors do play a role in the restoration of male copulatory behavior, based on the fact that blocking brain androgen receptors, blocks the restoration of male copulatory behavior.

The purpose of this series of studies with hydroxyflutamide was to test whether the administration of an antiandrogen combined with the administration of an antiestrogen would have a greater effect on hormone-dependent sociosexual behaviors than with the administration of either an antiandrogen or an antiestrogen alone. The studies in this chapter have been divided into two sections because different drug delivery methods were used. In Part 1, hydroxyflutamide was delivered directly into the brain by intracerebroventricular infusion (ICV) to test the hypothesis that blocking brain androgen receptors would inhibit the restoration of male copulatory behavior and partner preference. In Part 2, hydroxyflutamide was systemically administered by mini-osmotic pumps with and without injections of the antiestrogen RU 58668 to test the hypothesis that inhibition of both cell nuclear androgen and estrogen receptors would be more effective in blocking the restoration of several hormone-dependent sociosexual behaviors than inhibiting either receptor alone. Different drug delivery methods were used because the experimental design of the experiment changed as a result of switching antiestrogens (i.e., from ICI 182780 to RU 58668). The reason the antiestrogen was switched has already been discussed in the previous chapter, but briefly, since RU 58668 was found to be able to cross the blood-brain barrier, ICV drug delivery was not a necessity. By the time the experimental protocol was changed, the pilot studies with ICV delivery of the aromatase inhibitor fadrozole, the antiestrogen ICI 182780, and the antiandrogen hydroxyflutamide had already been completed.

Part 1: Effects of Intracerebroventricular Infusion of the Antiandrogen Hydroxyflutamide on Male Copulatory Behavior and Partner Preference

Introduction

The hypothesis for this experiment was that intracerebroventricular (ICV) delivery of the antiandrogen hydroxyflutamide would block the restoration of male copulatory behavior and partner preference. Administration of hydroxyflutamide (either by daily subcutaneous injections (McGinnis and Mirth 1986) or by intracranial implants (McGinnis et al. 1996)) has been previously shown to block the restoration of male copulatory behavior in rats. What made this study different was that the antiandrogen was delivered directly into the ventricular system of the brain by constant infusion, rather than by daily subcutaneous (sc) injections or implants into discrete brain regions. One potential advantage of ICV delivery was that brain cell nuclear androgen receptors could be blocked without targeting specific brain regions. Therefore, if multiple brain regions were mediating the effects of androgens in restoring male copulatory behavior, they all could be blocked at the same time, without affecting peripheral cell nuclear androgen receptors.

Experimental Design

The primary reason ICV delivery was chosen for this experiment was because I wanted to test whether simultaneously blocking both brain androgen and estrogen receptors would have a greater effect on blocking the restoration of male copulatory behavior and partner preference than blocking either receptor alone. ICV delivery was necessary for this experiment because the antiestrogen I originally intended to use (ICI 182780) could not penetrate the blood-brain barrier. This is why the first parts of Chapters 4 and 5 also begin with similar experiments. However, since the antiestrogen ICI 182780 did not have any

effect on the restoration of male copulatory behavior and the aromatase inhibitor fadrozole completely blocked the restoration of male copulatory behavior, the experiment where combinations of fadrozole, ICI 182780, and hydroxyflutamide were to be administered together was never performed.

The experimental design for this experiment was identical to the fadrozole and ICI 182780 pilot studies discussed in Chapters 3 and 4, respectively. The only difference was that instead of fadrozole or ICI 182780, the antiandrogen hydroxyflutamide was used. Because this was a pilot study, only one male copulatory behavior test was given after two weeks of testosterone/antiestrogen treatment for all but the highest dose of hydroxyflutamide tested (2 mg/day ICV). Animals in the 2 mg/day ICV hydroxyflutamide group were also tested for partner preference. Neither 50 kHz ultrasonic vocalization or scent marking tests were performed. One day after the last behavior test, on the second week of treatment, cell nuclear androgen receptor assays were performed to assess the effects of hydroxyflutamide.

Methods

The methods used for animal preparation, behavioral testing, data collection, and the cell nuclear androgen receptor assays were the same as described in the General Methods Chapter. Miniature osmotic pumps fitted to brain infusion cannulas were used to deliver hydroxyflutamide into the right lateral ventricle for 14 days. The dose of hydroxyflutamide was based on calculations from a study showing that with ICV delivery, 100 fold lower drug concentrations were possible to achieve similar brain drug concentrations as were obtained with systemic drug infusion (Smits and Struyker-Boudier 1979). Since this was the first study where hydroxyflutamide was administered by the ICV route, several different doses were tested, ranging from 2.5 μg/day to 2 mg/day ICV.

Hydroxyflutamide was dissolved directly in propylene glycol for all but the 2 mg/day group. However, hydroxyflutamide was dissolved directly in polyethylene glycol 200 (PEG-200) in the animals that received 2 mg/day ICV hydroxyflutamide, because the drug was more soluble in this solvent (Will et al. 1980). This allowed for a higher dose to be administered than was possible with propylene glycol, and was why two different solvents were used. Control males (T group) were gonadectomized and implanted with two, 10 mm testosterone-filled Silastic capsules and miniature osmotic pumps filled with propylene glycol (or PEG-200, n = 3) that were connected to a cannula in the right lateral ventricle. Gonadectomized males who were not implanted with testosterone capsules were also used as negative controls for the cell nuclear androgen assays (GdX group). The data for the 2 mg/day hydroxyflutamide dose were analyzed separately from the lower doses of hydroxyflutamide because partner preference tests, in addition to male copulatory behavior tests, were only performed in the 2 mg/day dose. This was done so that the experimental males could be compared with control males that received the same type of behavioral testing (i.e., males that were only tested for male copulatory behavior were not compared with males receiving partner preference tests and male copulatory behavior tests, and vice versa).

Results

The effects of intracerebroventricular delivery of hydroxyflutamide on the restoration of male copulatory behavior are summarized in Table 13. ICV delivery of hydroxyflutamide did not have any effect on the percentage of males ejaculating with doses ranging from 2.5 µg/day to 2 mg/day ICV ($\chi^2(6, N = 39) = 3.238$, $p = 0.7784$). The data for the percentage of males mounting and the percentage of males intromitting were similar, so they are not shown. Analysis of individual measures of male copulatory behavior did

not reveal any significant trends that were dose-dependent (Appendix A, Table 20 and Table 21 for the 2 mg/day dose).

ICV delivery of hydroxyflutamide (2 mg/day) did not inhibit the restoration of partner preference as measured by the time spent with the nonreceptive female subtracted from the time spent with the receptive female (ANOVA F (2,44) = 0.448, p = 0.6417, Figure 34). Interestingly, the time the male spent in the empty chamber was significantly affected by ICV delivery of hydroxyflutamide (ANOVA F (2,44) = 3.218, p = 0.0496, Appendix A, Figure 58). Specifically, the males treated with hydroxyflutamide ICV spent significantly more time in the empty chamber than the testosterone-treated gonadectomized male on test #2 (ANOVA F (1,44) = 5.833, p = 0.0199). However, this was the first case during the course of this work that the time spent in the middle chamber was affected, and its significance is unclear. On the other hand, the frequency of boundary crossing during the partner preference test was not affected by ICV delivery of hydroxyflutamide (ANOVA F (2,44) = 1.339, p = 0.2727, Appendix A, Figure 59).

Cell nuclear androgen receptor assays were performed to determine if the behavioral results correlated with blocking brain androgen receptors. Table 14 shows the effects of ICV administration of hydroxyflutamide on brain (pooled amygdala, hypothalamus, preoptic area, and septum; ANOVA F (7,25) = 4.07, p = 0.0042) and pituitary (ANOVA F (7,25) = 3.90, p = 0.0053) cell nuclear androgen receptor binding. Post hoc testing with Dunnett's one-tailed test for group means less than a control (testosterone-treated gonadectomized males) showed that only the GdX and T & 2 mg/day hydroxyflutamide groups were significantly different ($p < 0.05$) from the testosterone group in the brain. In the pituitary, the only group that was significantly different from the testosterone group was the GdX group. In addition, seminal vesicle weights were measured at the time of the assays to provide another measure of peripheral androgen receptor action in the GdX, T, T & 250 µg/day, T & 750 µg/day, and T & 2 mg/day groups (Figure 35, ANOVA F (4,42) = 28.69, p = 0.0001). Seminal vesicle weights were significantly reduced relative to the T

group in the gonadectomized males (which served as negative controls) and the groups with the two highest doses of hydroxyflutamide (750 µg/day and 2 mg/day).

Table 13. Effects of ICV Delivery of Hydroxyflutamide on the Percentage of Males Ejaculating

Group			Percentage of Males Ejaculating (test #2)	*n*
Testosterone (T)			92%	12
T & hydroxyflutamide	2.5	µg/day ICV	75%	4
T & hydroxyflutamide	12.5	µg/day ICV	100%	4
T & hydroxyflutamide	25.5	µg/day ICV	100%	4
T & hydroxyflutamide	250.0	µg/day ICV	75%	4
T & hydroxyflutamide	750.0	µg/day ICV	100%	3
T & hydroxyflutamide	2.0	mg/day ICV	88%	8

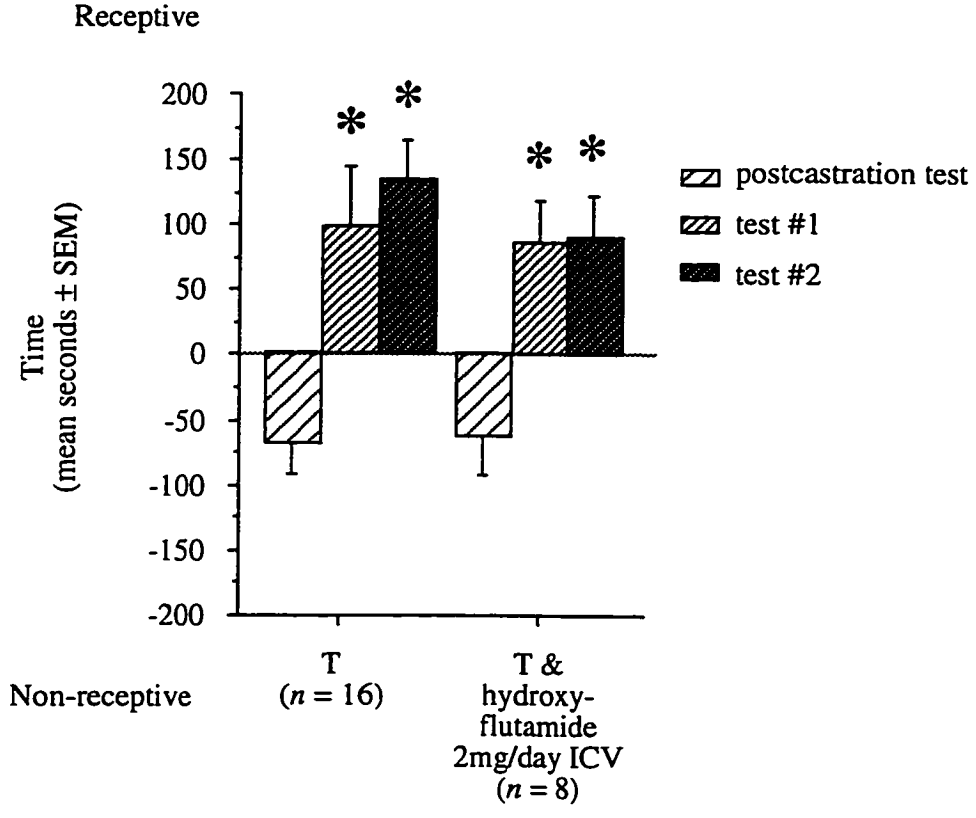


Figure 34. Effect of Intracerebroventricular Administration of Hydroxyflutamide on the Restoration of Partner Preference

Partner preference test (test #1, 7 days postcastration; test #2, 13 days postcastration) T = testosterone-treated gonadectomized males. The y-axis depicts time spent with the non-receptive female subtracted from time spent with the receptive female. Positive values indicate that more time was spent with the receptive female and vice versa. * $p < 0.05$, within group comparison vs. postcastration test, Least Square Mean Test

Table 14. Effect of Intracerebroventricular Administration of Hydroxyflutamide on Brain and Pituitary Nuclear Androgen Receptor Binding

Group			fmol AR/mg DNA (mean ± SEM) Brain				Pituitary				*n*
GdX			2.44	±	0.91	*	5.10	±	0.91	*	(7)
Testosterone (T)			44.46	±	6.97		78.75	±	16.50		(13)
T & hydroxyflutamide	2.5	µg/day ICV	28.84	±	17.85		135.90	±	20.60		(2)
T & hydroxyflutamide	12.5	µg/day ICV	56.02	±	24.48		121.80	±	7.04		(2)
T & hydroxyflutamide	25.5	µg/day ICV	61.15	±	24.12		115.17	±	6.04		(2)
T & hydroxyflutamide	250.0	µg/day ICV	35.89	±	2.82		62.98	±	0.69		(2)
T & hydroxyflutamide	750.0	µg/day ICV	22.99				71.02				(1)
T & hydroxyflutamide	2.0	mg/day ICV	13.63	±	5.00	*	73.00	±	9.66		(4)

* $p < 0.05$ vs. T, Dunnett's one-tailed test for means less than a control

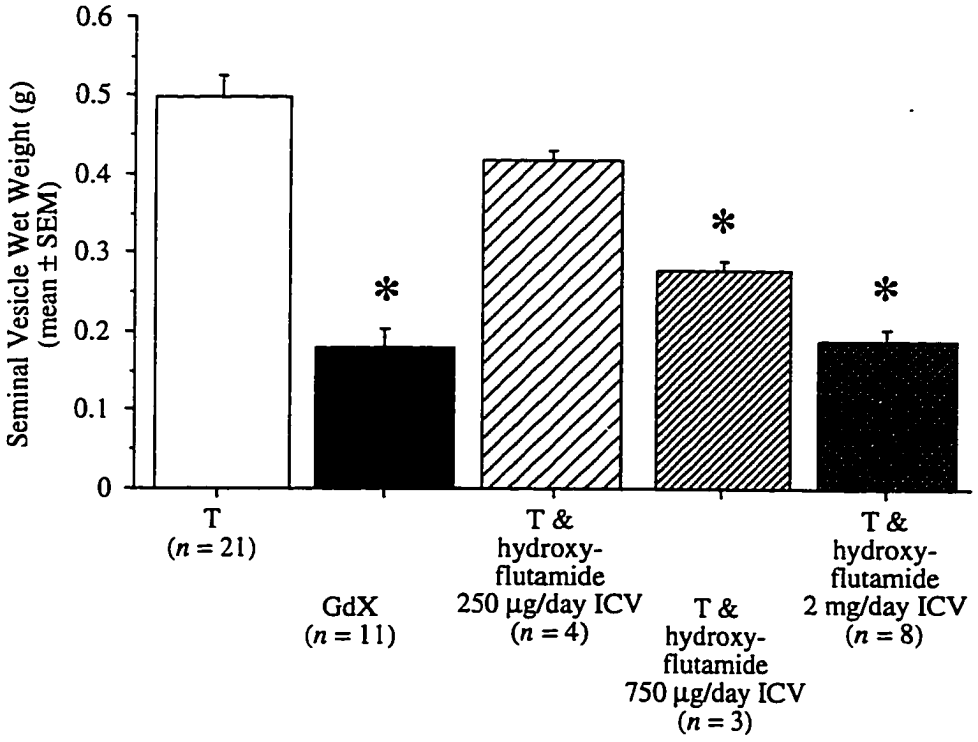


Figure 35. Effect of Intracerebroventricular Administration of Hydroxyflutamide on Seminal Vesicle Weights

* $p < 0.05$ vs. T, Dunnett's one-tailed test for means less than a control

Discussion

Intracerebroventricular (ICV) delivery of the antiandrogen hydroxyflutamide did not inhibit testosterone's restoration of male copulatory behavior in adult male rats. This result was surprising because previous studies had shown that this antiandrogen inhibited the restoration of male copulatory behavior when the drug was delivered systemically with daily injections (McGinnis and Mirth 1986) or when the drug was implanted directly into specific brain regions (McGinnis et al. 1996). The results from the cell nuclear androgen receptor assays shed some light on this discrepancy. For instance, at all but the highest dose of hydroxyflutamide given, there was no inhibition of brain or pituitary cell nuclear androgen receptor binding. Therefore, since it appears that brain androgen receptors were not blocked in all but the 2 mg/day group, it makes sense that there was no effect on the restoration of male copulatory behavior. However, brain androgen receptors were blocked in the 2 mg/day group, and yet there still was no inhibition of male copulatory behavior or partner preference. Doses of hydroxyflutamide higher than 2 mg/day for ICV delivery were not possible because at this dose the drug solution was nearly saturated.

One possible explanation for this inconsistency in the 2 mg/day group is that even though the results from the assay indicated that brain androgen receptor action was blocked, it is possible that there still was enough androgen receptor action in some brain regions to mediate the restoration of male copulatory behavior. The reason for this is that the assay used brain tissue from two rats consisting of pooled hypothalamus, preoptic area, amygdala, and septum. It is possible that pooling these brain regions diluted out differences in the amount of androgen receptor inhibition in different brain regions. So, even though the level of pooled brain androgen receptor was reduced, the amount of androgen receptor inhibition might not have been equivalent in all brain regions. The hypothesis that ICV administration of 2 mg/day hydroxyflutamide might not have reduced enough androgen

receptor action in a critical brain region is supported by the results from an intracranial implant study that showed the ability of hydroxyflutamide to block the restoration of male copulatory behavior was highly localized (McGinnis et al. 1996).

In addition, hydroxyflutamide implants into the ventromedial nucleus of the hypothalamus (VMN) block both male copulatory behavior and partner preference, but cannulas implanted less than 1 mm away from the VMN do not have any effect on the restoration of these behaviors (Marilyn McGinnis, unpublished observations). This indicates that an implant of hydroxyflutamide into the brain can block the restoration of both male copulatory behavior and partner preference in rats. However, the antiandrogen-filled intracranial implants must not be too far away from critical androgen receptor-containing brain regions in order to be effective in blocking male copulatory behavior. Therefore, ICV delivery of hydroxyflutamide might not have provided sufficient amounts of the antiandrogen to critical brain regions necessary for blocking testosterone's restoration of male copulatory behavior or partner preference.

Cell nuclear androgen receptor binding was measured in the pituitary (which lies outside the blood-brain barrier) for an index of peripheral androgen receptor action relative to brain androgen receptor action. The results from the androgen receptor assays indicate that at all the doses tested, pituitary androgen receptor binding was not affected by the ICV delivery of hydroxyflutamide. Seminal vesicle weights were also measured for an index on peripheral androgen receptor action because the weight of this tissue is highly dependent of the effects of androgens (Whalen and Edwards 1969). ICV delivery of hydroxyflutamide significantly reduced seminal vesicle weights in the 750 μg/day and 2 mg/day groups. This is interesting because this result indicates that the seminal vesicles are much more sensitive to the effects of an antiandrogen than can be measured with a nuclear exchange assay in the pituitary.

One aspect of this study was that ICV delivery provided a way to possibly block all aspects of brain androgen receptor action without affecting peripheral androgen receptors.

The actions of androgens in the periphery are thought to play an important role in the maintenance of sex structures such as the penis (McDonald et al. 1970), and there is a strong correlation between copulatory efficiency and the number of penile spines, which are androgen-dependent (Parrott 1975). In hamsters, peripheral androgenic stimulation is necessary for the restoration of male copulatory behavior (Lisk and Greenwald 1983). In rats, peripheral androgenic stimulation is also important, because males that receive anterior hypothalamus-preoptic area implants of estradiol are more likely to ejaculate when they also receive peripheral implants of dihydrotestosterone capsules (Davis and Barfield 1979).

Blocking brain androgen receptors with ICV delivery of hydroxyflutamide did not have any affect on the restoration of male copulatory behavior in the present study. This could be interpreted to mean that brain cell nuclear androgen receptors are not involved in mediating the restoration of male copulatory behavior in rats. However, intracranial implant studies in male rats indicate that the restoration of copulatory behavior can be inhibited by the site-specific delivery of hydroxyflutamide into the medial preoptic area or ventromedial hypothalamus (McGinnis et al. 1996). One reason for why ICV delivery of hydroxyflutamide did not impair the restoration of male copulatory behavior is that it is likely that not enough hydroxyflutamide can be delivered to critical brain sites by the ICV route. Therefore, the results from the present experiment cannot be used to argue the hypothesis that brain cell nuclear androgen receptors are not involved in the restoration of male copulatory behavior or partner preference.

Part 2: Effects of Combined Treatment with the Antiestrogen RU 58668 and the Antiandrogen Hydroxyflutamide on Hormone-Dependent Sociosexual Behaviors

Introduction

The experiments described up to this point have been focused on the roles of estradiol and estrogen receptor action in the restoration of hormone-dependent sociosexual behaviors. However, the results from these experiments have shown that testosterone (or one of its androgenic metabolites) is still necessary for mediating hormone-dependent sociosexual behaviors such as male copulatory behavior and partner preference. This is exemplified by the fact that physiological doses of estradiol alone do not fully restore male copulatory behavior (McGinnis et al. 1996, Vagell and McGinnis 1997). Therefore, the actions of both androgens and estrogens are necessary for the restoration of male copulatory behavior, but what are the mechanisms of action for these hormones?

As mentioned previously, the actions of steroid hormones on hormone-dependent sociosexual behaviors are believed to occur through the activation of classical nuclear steroid receptors (McEwen 1988). If this were true, blocking estrogen receptors, by treating males with an antiestrogen, should have inhibited the restoration of male copulatory behavior. However, the results discussed in the last chapter indicate that some of the actions of estradiol might not be mediated by brain cell nuclear estrogen receptors. This is because treatment with antiestrogens did not inhibit the restoration of male copulatory behavior in rats. Likewise, if androgen receptors mediate the effects of testosterone on male copulatory behavior in rats, blocking these receptors with an antiandrogen should also block the behavior. In spite of the fact that ICV delivery of hydroxyflutamide does not inhibit the restoration of male copulatory behavior (discussed in the previous section),

several studies using different methods of drug delivery have shown that hydroxyflutamide and its parent compound, flutamide, both inhibit the restoration of male copulatory behavior in rats (see Table 12 for references).

The purpose of this study was to assess the relative contributions of androgen receptor action and estrogen receptor action in the restoration of male copulatory behavior by the combined systemic administration of antagonists to androgen receptors and estrogen receptors. Even though the antiestrogen RU 58668 did not have any effect on the restoration of male copulatory behavior or partner preference, it is possible that androgen and estrogen receptor action could interact during the restoration of male copulatory behavior. When androgen receptors are active, more estradiol is produced from testosterone in the brain, because testosterone stimulates the induction of aromatase mRNA (resulting in increased aromatase protein levels and aromatase activity, Abdelgadir et al. 1994). So, blocking cell nuclear androgen receptors could make the animals more susceptible to the effects of blocking cell nuclear estrogen receptors with antiestrogens. This would be because the amount of estrogenic stimulation in the brain would be decreased from the lower levels of estradiol. In addition, the effect of antiandrogen treatment, with and without simultaneous antiestrogen treatment, on the restoration of partner preference, 50 kHz ultrasonic vocalizations, and scent marking were observed to test the hypothesis that these hormone-dependent sociosexual behaviors were differentially mediated by androgen receptor action and estrogen receptor action.

Experimental Design

This experiment was based on a restoration paradigm for male copulatory behavior, and therefore, was similar to the previous studies in male rats. However, hydroxyflutamide was delivered in a different manner than in the previous section. Hydroxyflutamide was delivered with a miniature osmotic pump (Alzet model 2ML2) implanted subcutaneously on

the animal's back. A dose of 15 mg/day (approximately 40 mg/kg) was used. Daily injections of this dose had been previously shown to inhibit the restoration of male copulatory behavior in rats (McGinnis and Mirth 1986). Hydroxyflutamide was delivered subcutaneously by constant infusion with miniature osmotic pumps in order to avoid fluctuations in drug levels during the 24 hour interval between injections. This was because hydroxyflutamide is rapidly degraded to inactive metabolites in the liver (Brogden and Clissold 1989). One group of animals in this study also received daily injections of RU 58668 along with the hydroxyflutamide to test the hypothesis that simultaneous inhibition of androgen and estrogen receptors would have a greater effect on blocking the restoration of male copulatory behavior than with either antagonist alone.

Methods

The methods used for animal preparation, behavioral testing, data collection and analysis, and cell nuclear estrogen receptor assays were the same as described in the General Methods Chapter. In addition to male copulatory behavior, partner preference and scent marking/ultrasonic vocalization tests were also included. Seminal vesicle weights were measured for an additional index of peripheral androgen receptor action along with the cell nuclear androgen receptor assays. Seminal vesicle weights were also used as a bioassay for whether the antiandrogen hydroxyflutamide actually blocked nuclear androgen receptor action. Data from animals that did not have significantly reduced seminal vesicle weights relative to testosterone-treated gonadectomized males were excluded from all analyses. *Note*: In the T & hydroxyflutamide & RU 58668 group, four animals did not have reduced seminal vesicle weights (mean ± SEM, 0.48 ± 0.07 g vs. 0.50 ± 0.03 g for the testosterone-treated gonadectomized males) at the time of the assays, and were excluded from the study. This reduced the number of animals in the T & hydroxyflutamide & RU 58668 group from $n = 10$ to $n = 6$.

Results

The effects of hydroxyflutamide (n = 10) and the combined administration of hydroxyflutamide and RU 58668 (n = 6) on the restoration of male copulatory behavior, as measured by the percentage of males ejaculating, are shown in Figure 36. Comparisons were made relative to testosterone-treated gonadectomized males (positive controls, n = 16). On test #1 (7 days after implant surgery), the T & hydroxyflutamide group was significantly different from the testosterone group (Fisher's Exact test (1, N = 26) p = 0.0036), but the T & hydroxyflutamide & RU 58668 group was not significantly different from the testosterone group (Fisher's Exact test (1, N = 22) $p > 0.9999$). On test #2 (13 days after implant surgery), both the T & hydroxyflutamide and the T & hydroxyflutamide & RU 58668 groups were significantly different from the testosterone group (Fisher's Exact test (1, N = 26) p = 0.0036 and Fisher's Exact test (1, N = 22) p = 0.0458, respectively). In addition, on test #2, there was no significant difference between the T & hydroxyflutamide and the T & hydroxyflutamide & RU 58668 groups (Fisher's Exact test (1, N = 16) $p > 0.6066$). However, there was a significant difference between the T & hydroxyflutamide and the T & hydroxyflutamide & RU 58668 groups on test #1 (Fisher's Exact test (1, N = 16) p = 0.0357). The data for the percentage of males mounting and the percentage of males intromitting were similar, and are not shown.

There were also significant differences in several individual measures of male copulatory behavior. Data from animals who copulated were analyzed with univariate repeated measures ANOVAs (Ekstrom et al. 1990). Multiple tests were used for each behavior to compare the experimental groups to the testosterone-treated gonadectomized males (positive controls). These correlated tests were used because post hoc tests, which take interaction effects into account, were not available for univariate repeated measures tests. In order to avoid inflating the type I error level, α was reduced from $p < 0.05$ to $p < 0.025$, since two tests were performed (Holm 1979). Also, this type of analysis did not

exclude rows of data that contained empty cells. This was useful because many animals who copulated on test #2 (13 days after implant surgery) did not copulate on test #1 (7 days after implant surgery). Therefore, data from every animal were used in the tests.

Mount frequency (Figure 37) was significantly increased in the T & hydroxyflutamide & RU 58668 group (ANOVA F (2,25) = 4.695, p = 0.0186), but not in the group treated with just T & hydroxyflutamide (ANOVA F (2,25) = 6.218, p = 0.0333, *note*: α was reduced from $p < 0.05$ to $p < 0.025$ for these comparisons). Postejaculatory interval (Figure 39) was also significantly increased in the group treated with T & hydroxyflutamide & RU 58668 (ANOVA F (2,23) = 6.295, p = 0.00066), but not in the group treated with the T & hydroxyflutamide (ANOVA F (2,31) = 1.743, p = 0.1978). On the other hand, hit rate (Figure 38) was significantly decreased in the group treated with T & hydroxyflutamide & RU 58668 (ANOVA F (2,29) = 7.050, p = 0.0032), and in the group treated with T & hydroxyflutamide (ANOVA F (2,23) = 8.835, p = 0.0014). In the corresponding figures, specific group differences for each test relative to the T group are indicated by asterisks.

Partner preference was significantly decreased relative to the T group in both the T & hydroxyflutamide group and T & hydroxyflutamide & RU 58668 group (ANOVA F (4,58) = 4.529, p = 0.0030, Figure 40). In the group treated with T & hydroxyflutamide & RU 58668 (ANOVA F (2,58) = 4.395, p = 0.0167) and in the group treated with T & hydroxyflutamide (ANOVA F (2,58) = 7.408, p = 0.0014), the amount of time spent with the nonreceptive female subtracted from the amount of time spent with the receptive female was significantly lower than in the T group. However, neither the time the male spent in the empty chamber nor the frequency of boundary crossing during the partner preference test were significantly affected by either experimental treatment (ANOVA F (2,58) = 0.873, p = 0.4860, Appendix A, Figure 60; and ANOVA F (2,58) = 0.865, p = 0.4902, Appendix A, Figure 61, respectively).

The effects of hydroxyflutamide alone or hydroxyflutamide and RU 58668 on testosterone's restoration of 50 kHz vocalizations and scent marking are depicted for each individual animal in Appendix A, Figure 62 and Figure 63, respectively. These figures illustrate the large amount of interanimal and intertest variability in this data. Therefore, the statistical analysis was performed on the paired difference scores between tests as described in the General Methods Chapter, rather than on the raw data. Treatment with hydroxyflutamide alone or hydroxyflutamide and RU 58668 significantly impaired the restoration of 50 kHz vocalizations during the first week of testosterone treatment (ANOVA F (2,22) = 7.850, p = 0.0027, Figure 41A). However, only the combined administration of both hydroxyflutamide and RU 58668 significantly impaired the restoration of 50 kHz vocalizations during the second week of testosterone treatment (ANOVA F (2,22) = 3.702, p = 0.0411, Figure 41B). With respect to scent marking, treatment with hydroxyflutamide alone or hydroxyflutamide and RU 58668 significantly impaired testosterone's restoration of scent marking during the first week of treatment (test #1 - postcastration test, ANOVA F (2,22) = 8.151, p = 0.0022, Figure 42A), but not during the second week of treatment (test #2 - test #1, ANOVA F (2,22) = 0.168, p = 0.8466, Figure 42B). However, one reason there was no effect of antiandrogen or combined antiandrogen and antiestrogen treatment during the second week of treatment was that there was no increase in scent marking during the second week of testing in the positive controls.

Cell nuclear androgen receptor assays were performed to determine if hydroxyflutamide blocked brain and pituitary androgen receptors. These data are shown in Figure 43 for brain tissue (pooled hypothalamus, preoptic area, amygdala, and septum; ANOVA F (2, 22) = 13.874, p = 0.0001) and in Figure 44 for pituitary (ANOVA F (3, 26) = 6.38, p = 0.0022). Gonadectomized males had significantly lower levels of cell nuclear androgen receptor binding than gonadectomized males treated with testosterone in both the brain (p = 0.0001) and pituitary (p = 0.0008). Subcutaneous delivery of hydroxyflutamide with miniature osmotic pumps (T & hydroxyflutamide group) significantly reduced the

level of brain (p = 0.0014) and pituitary (p = 0.0107) cell nuclear androgen receptor binding relative to gonadectomized males treated with testosterone (T group, positive controls).

The combined administration of hydroxyflutamide and RU 58668, in gonadectomized males treated with testosterone, significantly decreased (p = 0.0097) pituitary cell nuclear androgen receptor binding relative to gonadectomized males treated with testosterone, and was also not significantly different (p = 0.5723) from the level in gonadectomized males. The level of brain cell nuclear androgen receptor binding in the hydroxyflutamide and RU 58668 treated males was not measured because the tissue in these animals was used for brain cell nuclear estrogen receptor binding assays (ANOVA F (2, 21) = 9.147, p = 0.0014, Figure 45). The combined administration of hydroxyflutamide and RU 58668 significantly decreased (p = 0.0240) brain cell nuclear estrogen receptor binding relative to gonadectomized males treated with testosterone, and was not significantly different (p = 0.2689) from the level in gonadectomized males.

For another index of peripheral androgen receptor action, seminal vesicle weights were measured at the time of the assays (ANOVA F (3, 44) = 127.220, p = 0.0001, Figure 46). Seminal vesicles from gonadectomized males weighed significantly less (p = 0.0001) than seminal vesicles from gonadectomized males treated with testosterone. Hydroxyflutamide alone (p = 0.0001) or hydroxyflutamide and RU 58668 (p = 0.0001) also significantly reduced seminal vesicle weights in gonadectomized males treated with testosterone to levels that were the same as gonadectomized males (p = 0.2511 and p = 0.1699, respectively).

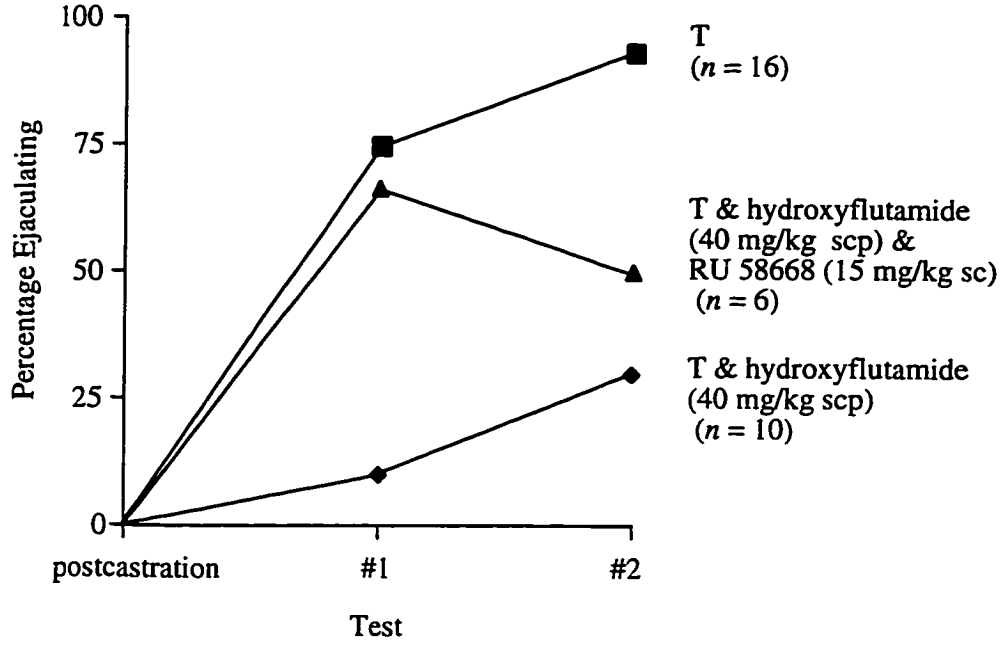


Figure 36. Effects of Hydroxyflutamide and RU 58668 on the Percentage of Males Ejaculating

Test #1 was 7 days after implant surgery and test #2 was 13 days after implant surgery. The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. On test #1, the T & hydroxyflutamide group was significantly different from the testosterone group (Fisher's Exact test, $p = 0.0036$), but the T & hydroxyflutamide & RU 58668 group was not significantly different from the testosterone group. On test #2, both the T & hydroxyflutamide and the T & hydroxyflutamide & RU 58668 groups were significantly different from the testosterone group (Fisher's Exact test, $p = 0.0053$ and $p = 0.0458$, respectively).

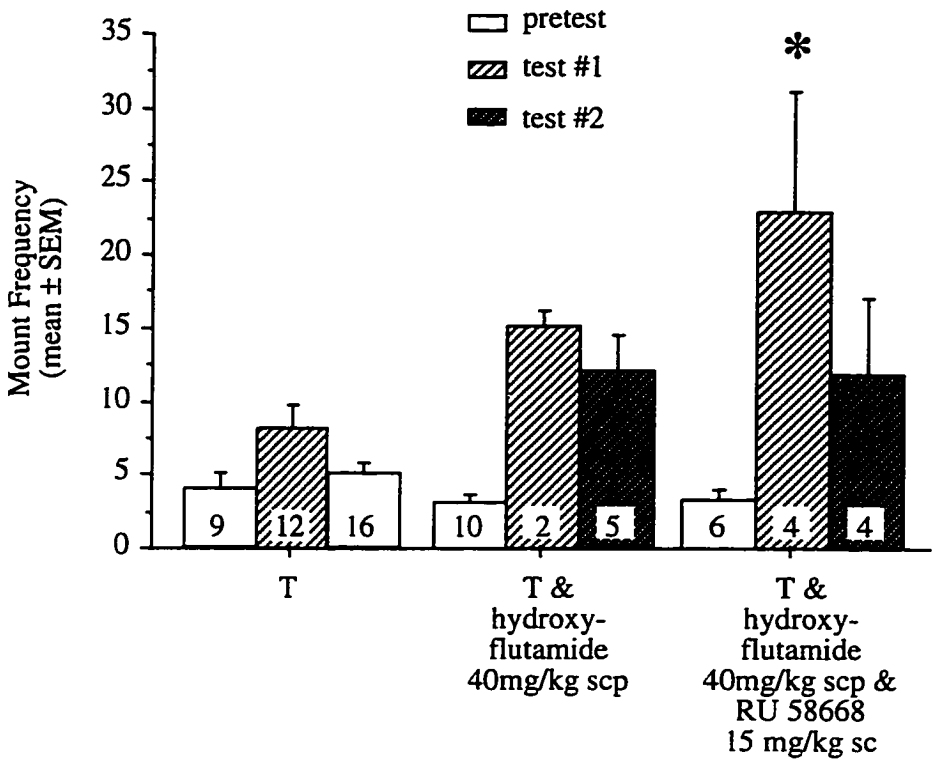


Figure 37. Effects of Hydroxyflutamide and RU 58668 on Mount Frequency

The digits in the columns show the number of animals behaving on each test (*n*). The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. In the T group, some animals were not recorded during the pretest. This is why the number of animals for test #2 is greater than the number of animals behaving on the pretest for the T group. The asterisk indicates $p < 0.05$ vs. the T group on the same test day, Least Square Mean Test.

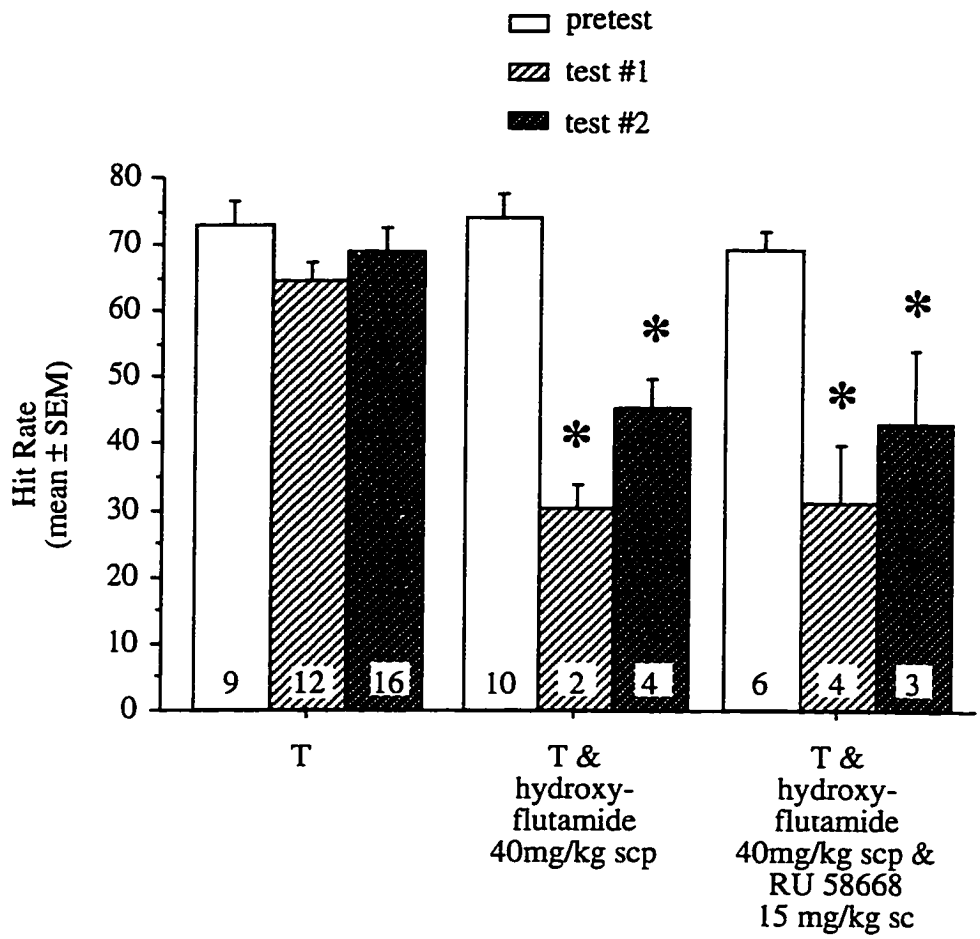


Figure 38. Effects of Hydroxyflutamide and RU 58668 on Hit Rate

The digits in the columns show the number of animals behaving on each test (*n*). The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. In the T group, some animals were not recorded during the pretest. This is why the number of animals for test #2 is greater than the number of animals behaving on the pretest for the T group. The asterisk indicates $p < 0.05$ vs. the T group on the same test day, Least Square Mean Test.

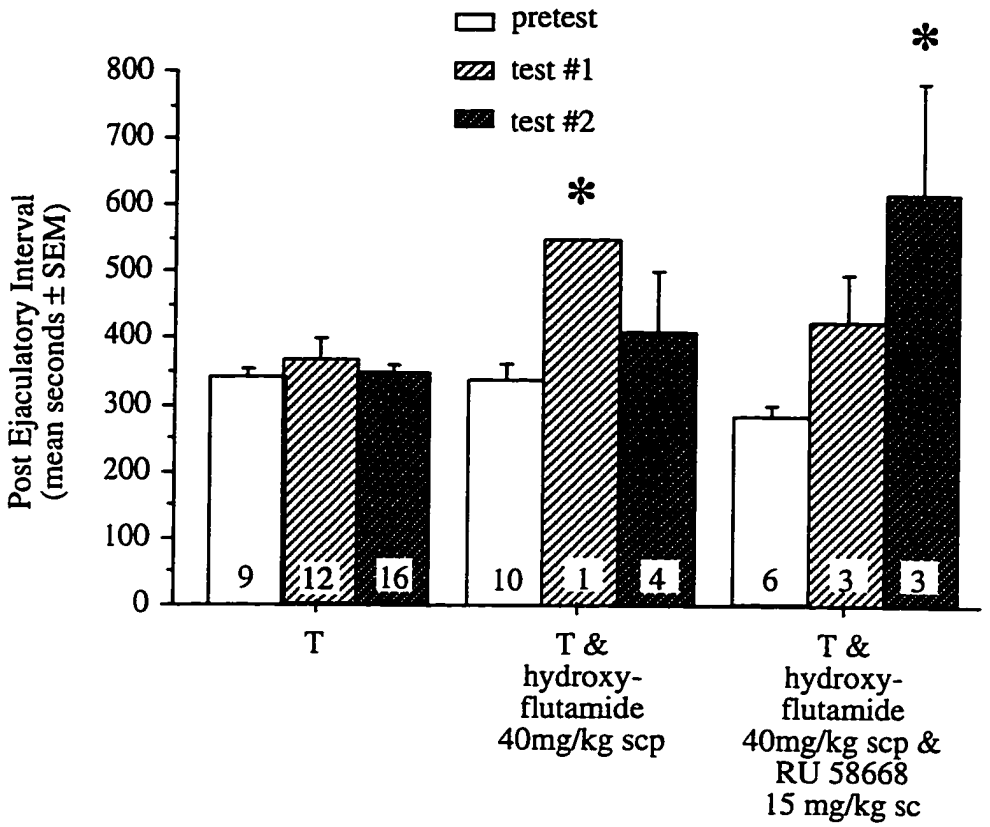


Figure 39. Effects of Hydroxyflutamide and RU 58668 on Postejaculatory Interval

The digits in the columns show the number of animals behaving on each test (*n*). The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. In the T group, some animals were not recorded during the pretest. This is why the number of animals for test #2 is greater than the number of animals behaving on the pretest for the T group. The asterisk indicates $p < 0.05$ vs. the T group on the same test day, Least Square Mean Test.

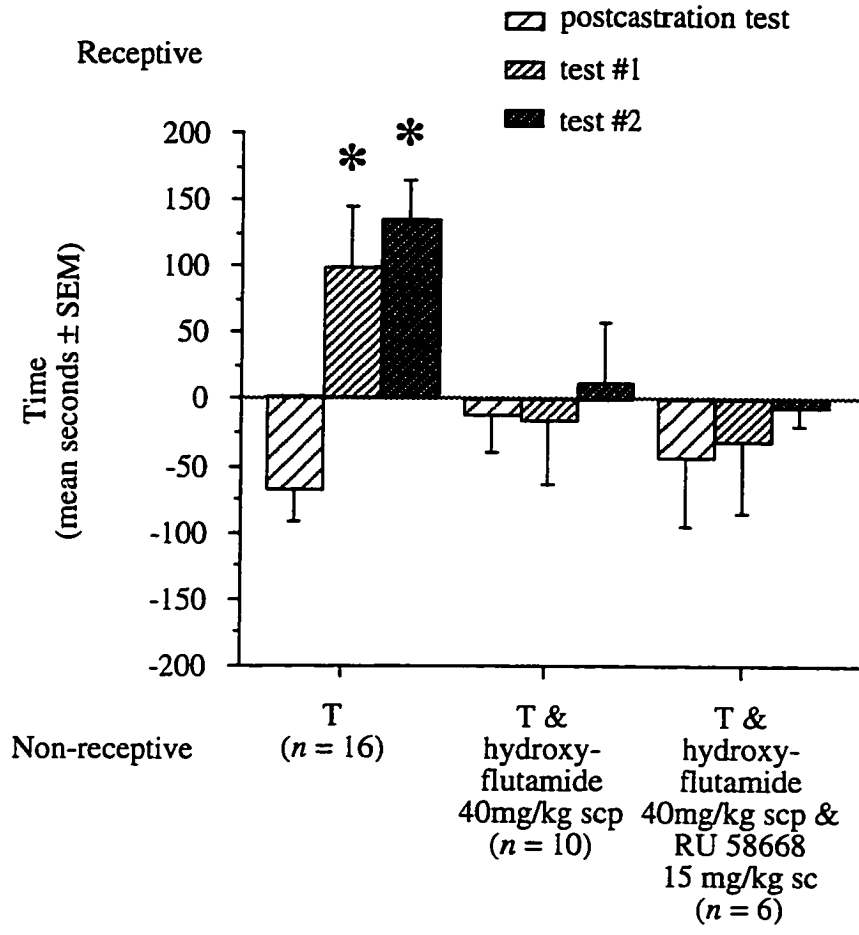


Figure 40. Effects of Hydroxyflutamide and RU 58668 on the Restoration of Partner Preference

Partner preference test (test #1, 7 days postcastration; test #2, 13 days postcastration) T = testosterone-treated gonadectomized males. The y-axis depicts time spent with the non-receptive female subtracted from time spent with the receptive female. Positive values indicate that more time was spent with the receptive female and vice versa. The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. * $p < 0.05$, within group comparison vs. postcastration test, Least Square Mean Test

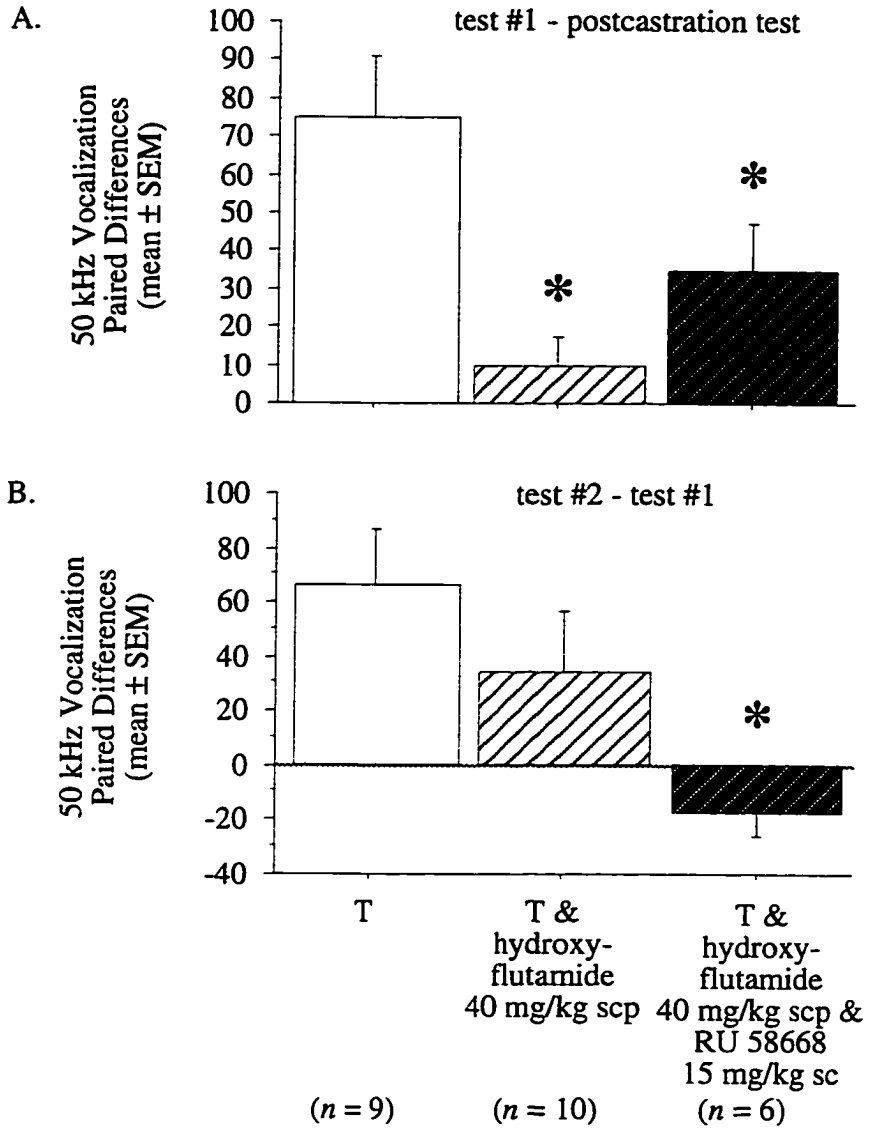


Figure 41. Effects of Hydroxyflutamide and RU 58668 on the Restoration of 50 kHz Vocalizations

The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. * $p < 0.05$ vs. T, Fisher's PLSD post hoc test

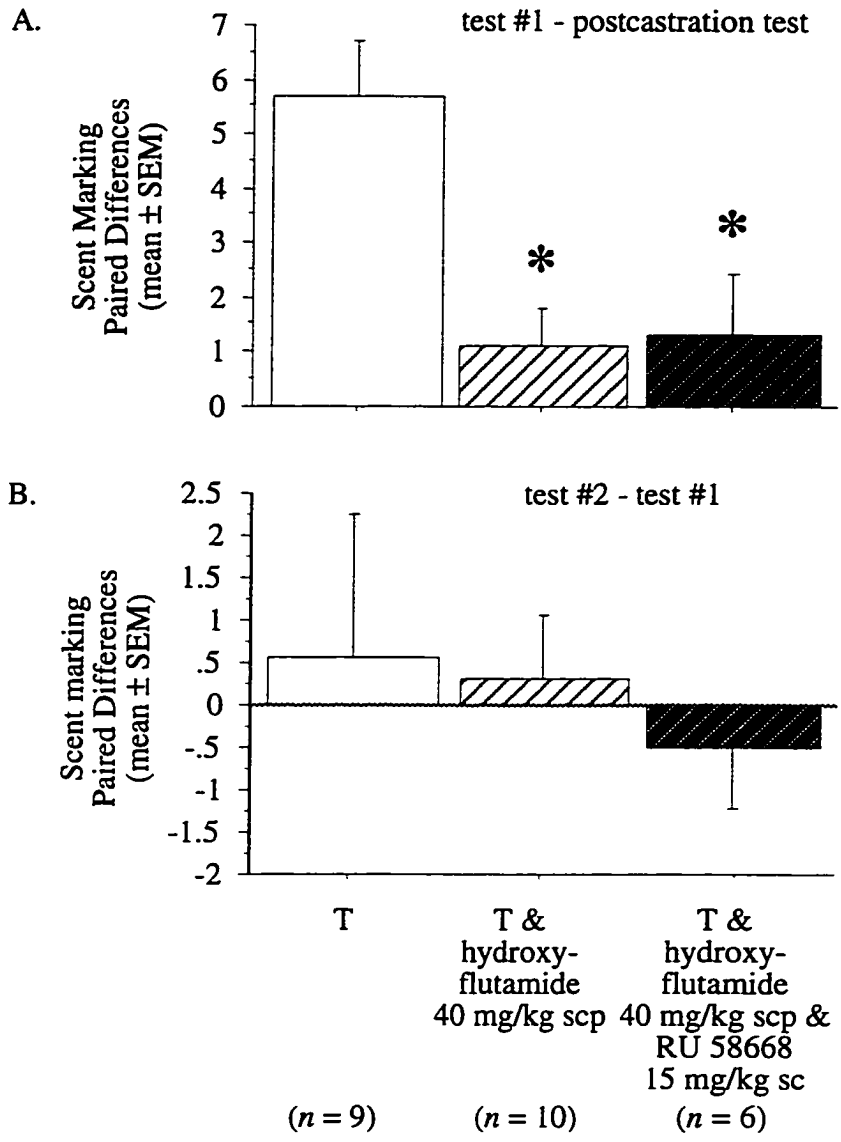


Figure 42. Effects of Hydroxyflutamide and RU 58668 on the Restoration of Scent Marking

The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. * $p < 0.05$ vs. T, Fisher's PLSD post hoc test

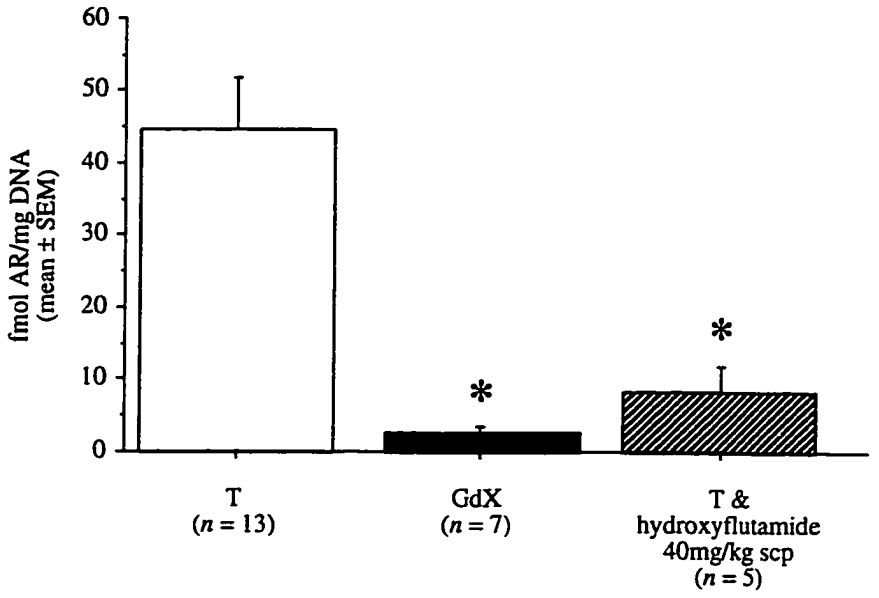


Figure 43. Effects of Hydroxyflutamide and RU 58668 on Brain Cell Nuclear Androgen Receptor Binding

Nuclear androgen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala). The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. T = gonadectomized males implanted with testosterone capsules GdX = gonadectomized males * $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

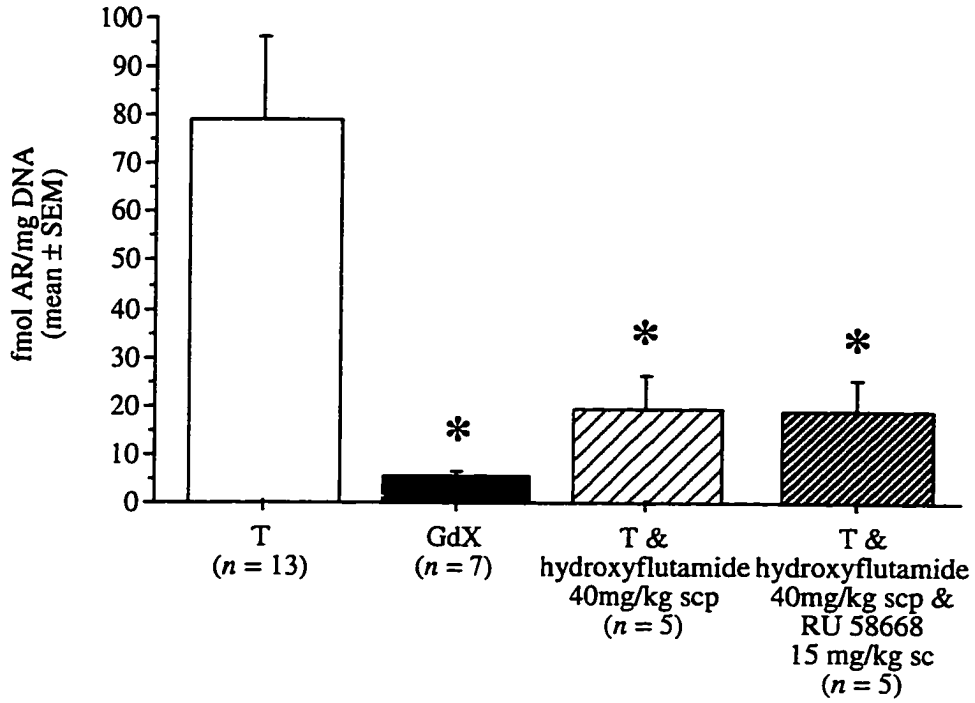


Figure 44. Effects of Hydroxyflutamide and RU 58668 on Pituitary Cell Nuclear Androgen Receptor Binding

Nuclear androgen receptor exchange assays in pituitary. The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. T = gonadectomized males implanted with testosterone capsules GdX = gonadectomized males
* $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

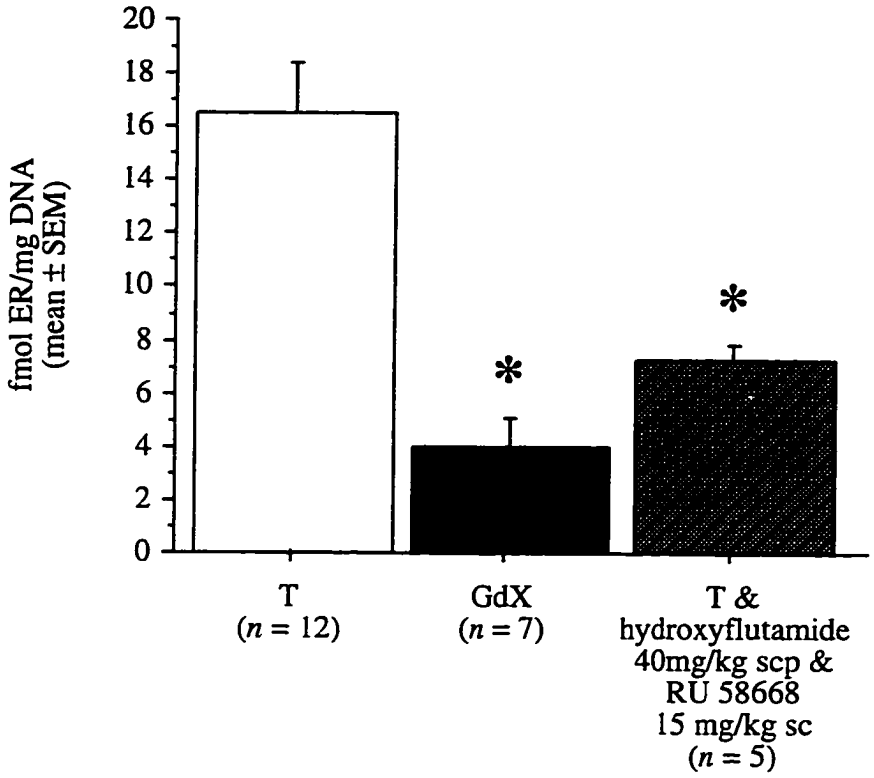


Figure 45. Effects of Hydroxyflutamide and RU 58668 on Brain Cell Nuclear Estrogen Receptor Binding

Nuclear estrogen receptor exchange assays in brain (pooled preoptic area, hypothalamus, septum, and amygdala). The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. T = gonadectomized males implanted with testosterone capsules GdX = gonadectomized males * $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

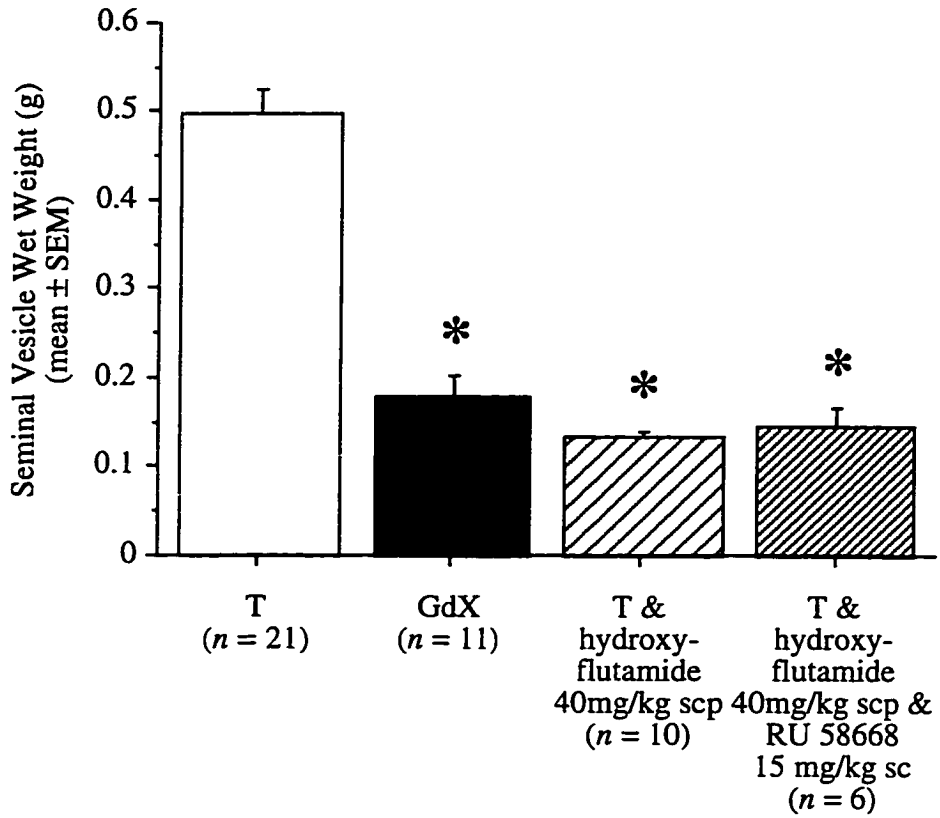


Figure 46. Effects of Hydroxyflutamide and RU 58668 on Seminal Vesicle Weights

The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively. T = gonadectomized males implanted with testosterone capsules GdX = gonadectomized males
* $p < 0.05$ vs. T, Dunnett's two-tailed post-hoc test

Discussion

The antiandrogen hydroxyflutamide significantly reduced the percentage of males mounting, intromitting, and ejaculating during the restoration of male copulatory behavior in testosterone-treated gonadectomized male rats. These data are consistent with previous studies, which showed that nonsteroidal antiandrogens decreased the percentage of males mounting, intromitting, and ejaculating (Gladue and Clemens 1980, McGinnis and Mirth 1986). The hypothesis that chronic infusion of hydroxyflutamide would be more effective in inhibiting the restoration of male copulatory behavior than daily injections was not tenable, because chronic infusion with subcutaneous miniature osmotic pumps did not appreciably inhibit the restoration of male copulatory behavior more than was observed previously with daily injections (McGinnis and Mirth 1986).

Besides reducing the percentage of males performing different behaviors, treatment with testosterone and hydroxyflutamide also resulted in deficits in two closely related aspects of male copulatory behavior. Males treated with testosterone and hydroxyflutamide mounted more, but did not intromit more, than testosterone-treated gonadectomized male rats. Consequently, there was a decrease with the hit rate in the male treated with testosterone and hydroxyflutamide. Hit rate is the number of mounts relative to the total number of mounts and mounts with intromission, and has been used as a measure of copulatory performance (Gray 1977, Pfaus et al. 1990). These data are in agreement with three previous studies (Sodersten et al. 1975, Gray 1977, Gladue and Clemens 1980), which showed that males treated with flutamide displayed deficits in copulatory performance (measured as the hit rate). Moreover, males treated with estradiol alone (discussed in Chapter 4) mounted significantly more than control males treated with testosterone (see Appendix A, Table 17). This is interesting because males treated with estradiol alone can be thought of as having a maximum level of androgen receptor

inhibition, since they did not have any testosterone in the first place. Therefore, these studies indicate that cell nuclear androgen receptors play an important role in restoring copulatory performance. Moreover, it is likely that the sites where cell nuclear androgen receptors are playing a role in modulating copulatory performance are in the brain. The evidence for this hypothesis is that male rats, implanted with cannulas filled with testosterone propionate into the medial preoptic area, display characteristics of pelvic thrusting that are similar to intact (control) males (Morali et al. 1986).

Treatment with the antiestrogen RU 58668 along with testosterone and the antiandrogen hydroxyflutamide did not result in a synergistic suppression of the restoration of male copulatory behavior, beyond what was observed with animals treated with just testosterone and hydroxyflutamide. Out of all the aspects of male copulatory behavior examined in this study, only postejaculatory interval was significantly increased by treatment with T & hydroxyflutamide & RU 58668, even though treatment with T & hydroxyflutamide was not significantly different relative to testosterone-treated gonadectomized male rats. However, this result was from an *n* of 3, and was only significant on the second week of testing. Therefore, conclusions based on this result for postejaculatory interval need to be viewed with caution, since no other aspect of male copulatory behavior was affected in a similar manner.

This was one of the first studies to test the hypothesis that the restoration of partner preference could be blocked by treatment with an antiandrogen and/or an antiestrogen. In this work, the restoration of partner preference was completely inhibited in testosterone-treated gonadectomized male rats receiving either hydroxyflutamide or hydroxyflutamide and RU 58668. There was no difference in the restoration of partner preference between the group receiving the antiandrogen alone or the group receiving both the antiandrogen and antiestrogen. The antihormones reduced the amount of time the male spent with the receptive female, but the extra time remaining in the test was divided between the empty compartment and with the nonreceptive female. Neither treatment affected the number of

boundary crossings, so the animals had similar activity levels. These data are in agreement with a study showing that hydroxyflutamide implants into the ventromedial nucleus of the hypothalamus (VMN) can also block partner preference in adult male rats (Marilyn McGinnis, unpublished observations). Therefore, sexual motivation, as measured in the partner preference test (Everitt 1990), is an androgen receptor-dependent behavior that is independent of cell nuclear estrogen receptors.

The present study was also the first to test the hypothesis that the restoration of 50 kHz vocalizations and scent marking could be inhibited with antiandrogen and/or antiestrogen treatment in adult male rats. The study of how steroid hormones mediate the restoration of 50 kHz vocalizations and scent marking is relatively new in rats, as compared to male copulatory behavior. Consequently, there are few other studies in rats (Matochik and Barfield 1991, Matochik and Barfield 1994) to compare the present results with, and these other studies examined the roles of steroid hormones, not the roles of steroid hormone receptors, in mediating these behaviors. It has been reported that some male rats do not show declines in 50 kHz vocalizations after gonadectomy (Matochik and Barfield 1994). However, the majority of animals in the present study displayed significant declines in 50 kHz vocalizations two weeks after gonadectomy, although there was a high amount of interanimal variability (this work was discussed in Chapter 3). To address the problem of interanimal variability, the present data were analyzed with one-way ANOVAs on the paired difference scores from each animal. This provided that a way to normalize the scores from each animal, so that treatment differences across the groups could be compared.

Matochik and Barfield (1991) found that neither estradiol implants alone nor dihydrotestosterone implants alone were sufficient to restore 50 kHz vocalizations as well as testosterone did. However, Matochik and Barfield (1991) did not examine the effects of combined treatment with dihydrotestosterone and estradiol. Their results showed that only an aromatizable androgen, which would supply both androgens and estrogens, was sufficient for the restoration of 50 kHz vocalizations. In the present study, a surprising

temporal pattern emerged with 50 kHz vocalizations. During the first week of testing after implant surgery, treatment with hydroxyflutamide alone or both hydroxyflutamide and RU 58668 blocked the restoration of 50 kHz vocalizations. However, during the second week of testing, only the combined administration of hydroxyflutamide and RU 58668 blocked the restoration of 50 kHz vocalizations. Treatment with RU 58668 alone also blocked testosterone's restoration of 50 kHz vocalizations during the second week of treatment (discussed in Chapter 5, Part 4). This indicated that the antiandrogen was effective in inhibiting the restoration of 50 kHz vocalizations during the first week of testing, but that the antiestrogen was effective in inhibiting the restoration of 50 kHz vocalizations during the second week of testing. Therefore, both cell nuclear androgen receptors and estrogen receptors were involved in restoring this behavior, but these receptors were involved at different times during the restoration of 50 kHz vocalizations.

In contrast to 50 kHz vocalizations, which increased during the two weekly intervals between the tests, the restoration of scent marking was complete after one week of testosterone treatment. This observation that the restoration of scent marking was complete after 1 week of testosterone treatment was not consistent with the results of Matochik and Barfield (1991), who found that there was roughly a linear increase in scent marking over 30 days after testosterone capsules were implanted. Matochik and Barfield (1991) found that the restoration of scent marking was restored with estradiol or testosterone, but not with dihydrotestosterone. This implied that the actions of estradiol were sufficient for the restoration of scent marking. However, Matochik's and Barfield's (1991) data did not preclude a possible role for androgens in the restoration of scent marking, only that dihydrotestosterone was not sufficient to restore scent marking. Moreover, testosterone and dihydrotestosterone might not be equivalent in their ability to activate androgen receptors (Deslypere et al. 1992).

Both RU 58668 (discussed in Chapter 5, Part 4) and hydroxyflutamide (present study) impaired testosterone's restoration of scent marking. However, treatment with

hydroxyflutamide was better at inhibiting the restoration of scent marking than treatment with RU 58668 was. There was no additive effect on the inhibition of scent marking with combined hydroxyflutamide and RU 58668 treatment. The reason why hydroxyflutamide inhibited testosterone's restoration of scent marking, even though treatment with dihydrotestosterone did not restore this behavior is unknown; especially since estradiol restored this behavior (Matochik and Barfield 1991). One possible mechanism, which could explain these results, is that while the actions of both androgens and estrogens are necessary for the restoration of scent marking, enough estradiol stimulation might be sufficient to restore this behavior in the absence of gonadal androgens. Another possibility is that adrenal steroids (such as dehydroepiandrosterone [DHEA]) might be sufficient to serve as precursors for the synthesis of gonadal androgens when estradiol is administered to gonadectomized male rats (Labrie et al. 1993).

Data from the cell nuclear steroid receptor assays and the seminal vesicle weights support the hypothesis that the behavioral changes corresponding to the antihormone treatments coincided with reductions in steroid receptor action. Both the brain cell nuclear androgen receptor and estrogen receptor assays showed that treatment with the antihormones reduced the levels of the respective receptors to the levels found in gonadectomized males. Therefore, these antihormone treatments were able to achieve a maximal level of receptor inhibition, within the sensitivity of these techniques to measure. The results from the pituitary cell nuclear androgen receptor assay did not indicate that the combined administration of the antiandrogen, hydroxyflutamide, and the antiestrogen, RU 58668, affected the amount of cell nuclear androgen receptor binding in a way that was different from treatment with hydroxyflutamide alone.

The results from this study on the restoration of several sociosexual behaviors do not support the hypothesis that the combined inhibition of both cell nuclear androgen receptors and estrogen receptors is any different from blocking either of these receptors alone. However, not all hormone-dependent sociosexual behaviors are mediated by the

same mechanisms in adult male rats. This is evident in the fact that cell nuclear estrogen receptor action plays a role in the restoration of both 50 kHz vocalizations and scent marking, but not in the restoration of male copulatory behavior or partner preference. In conclusion, these results reinforce the notion that the restoration of male copulatory behavior is strongly influenced by cell nuclear androgen receptor action, but not by cell nuclear estrogen receptor action. This work provides evidence for a new, but unknown, role of estrogen action in adult male rats that is independent of cell nuclear estrogen receptors, and should serve to shift the focus from the actions of classical cell nuclear estrogen receptors to the study of other ways in which estrogen signaling occurs in the brain.

Chapter 7 - Conclusions

Summary

The overall goal of this project was to assess the role of aromatization and steroid receptor activation in the restoration of male rat sociosexual behavior. To that end, the experiments described in this dissertation involved attempts at blocking the restoration of different hormone-dependent sociosexual behaviors (such as male copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking) in adult male rats with inhibitors to aromatase, cell nuclear estrogen receptors, and cell nuclear androgen receptors (see Figure 47). The work was divided into four specific aims, which are summarized hereafter and in Table 15:

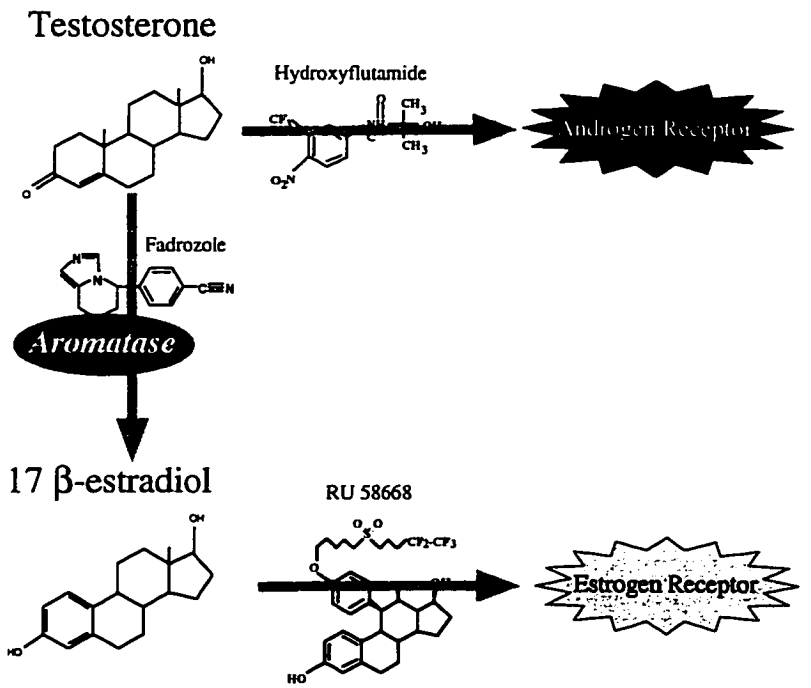


Figure 47. Inhibitors Used to Block Aromatase, Cell Nuclear Estrogen Receptors, and Cell Nuclear Androgen Receptors

Table 15. Restoration of Sociosexual Behaviors Blocked by the Different Inhibitors

	aromatase inhibitor	antiandrogen	antiestrogen	antiandrogen & antiestrogen
male copulatory behavior	√	√		√
partner preference		√		√
50 kHz vocalizations	nd	√	√	√
scent marking	nd	√	√	√

Note: The check marks indicate that the inhibitors impaired the restoration of the behaviors.
nd = not determined

Specific Aim #1:

To block testosterone's restoration of male copulatory behavior and partner preference by inhibiting brain aromatase.

This work assessed the role of aromatization in the expression of male reproductive behavior by testing the effects of the aromatase inhibitor, fadrozole, on the restoration of male copulatory behavior and partner preference in testosterone-treated gonadectomized rats. My results support the hypothesis that aromatization is necessary for the restoration of male copulatory behavior in rats. However, estradiol-treatment, without testosterone, was not sufficient for the restoration of male copulatory behavior. Therefore, both androgens and estrogens are necessary for the complete restoration of male copulatory behavior. On the other hand, aromatization was not necessary for the restoration of partner preference. So, the neural mechanisms that mediate male copulatory behavior and sexual motivation might be different. This work was described in Chapter 4.

Specific Aim #2:

To block testosterone's restoration of hormone-dependent sociosexual behaviors by directly inhibiting estrogen receptors with chronic antiestrogen treatment.

Estradiol's role in mediating male copulatory behavior has been widely accepted (reviewed in : Beyer et al. 1976a, Naftolin and MacLusky 1984). However, inadequate data has been used to support the hypothesis that cell nuclear estrogen receptors are involved. This could be because the nonsteroidal antiestrogens previously used, such as tamoxifen (Beyer et al. 1976b, McGinnis et al. 1990) and CI-628 (Luttge 1975), were partial agonists with a low affinity for estrogen receptors. The hypothesis that cell nuclear estrogen receptors mediated estradiol's effects in restoring hormone-dependent sociosexual behaviors was tested with the recently developed antiestrogens, ICI 182780 and RU 58668. These steroidal antiestrogens are not believed to posses any partial agonist activity, unlike the most of the antiestrogens currently available (Nicholson et al. 1996). This work began with the use of the antiestrogen, ICI 182780 (described in Chapter 5, Part 1), because more was known about it than any other "pure" antiestrogen at the time. However, ICI 182780 had no effect on the restoration of male copulatory behavior or on reducing nuclear estrogen receptor occupation levels. Therefore, another "pure" antiestrogen, RU 58668, was selected. Unfortunately, only a few papers concerning the effectiveness of RU 58668 had been published when this work began (Van de Velde et al. 1994, Jin et al. 1995, Montagne et al. 1995, Nique and Van de Velde 1995, Van de Velde et al. 1995), and none had tested for the compound's ability to block brain estrogen receptors. In addition, it was not known whether this antiestrogen blocked estrogen-dependent behaviors in female rats. Whether or not RU 58668 blocked estrogen-dependent behaviors in female rats was important because if RU 58668 did not block male copulatory behavior, it would not be known whether this was due to antiestrogen's effectiveness. Consequently, the

effects of RU 58668 on blocking several estrogen-dependent parameters in female rats was measured (described in Chapter 5, Part 3).

My experiments with RU 58668 in female rats indicated that it blocked several known estrogen-dependent behaviors and biochemical measures. In addition, RU 58668 did not affect cell nuclear androgen receptor occupation in male rats (described in Chapter 5, Part 4). Therefore, RU 58668 was used to study the role of estrogen receptor action in hormone-dependent sociosexual behaviors. Neither 3 mg/kg/day nor 15 mg/kg/day of RU 58668 had any effect on male copulatory behavior or partner preference, even though brain cell nuclear estrogen receptor occupation was significantly reduced to the level found in gonadectomized males (described in Chapter 5, Part 4). However, there was a substantial (but not significant) trend indicating that the restoration of scent marking was inhibited by both doses of RU 58668. In addition, the restoration of 50 kHz vocalizations was blocked by RU 58668 during the second week of testosterone treatment. Therefore, it is likely that there are diverse neural mechanisms mediating the restoration of different hormone-dependent behaviors. In conclusion, this work with estrogen receptor antagonists suggests that estradiol's effects on male copulatory behavior may not be mediated by cell nuclear estrogen receptors.

Specific Aim #3:

To block testosterone's restoration of hormone-dependent sociosexual behaviors by directly inhibiting androgen receptors with chronic antiandrogen treatment.

The role of androgen receptors in mediating male copulatory behavior had been well established using the antiandrogen, hydroxyflutamide (McGinnis and Mirth 1986). Constant intracerebroventricular (ICV) infusion of hydroxyflutamide did not have any effect on the restoration of male copulatory behavior even at the maximum ICV dose possible (described in Chapter 6, Part 1). The failure of ICV delivery of hydroxyflutamide

to inhibit the restoration of male copulatory behavior was probably due to a technical limitation of this method in delivering enough hydroxyflutamide to sufficiently block brain androgen receptors in critical brain regions, because subcutaneous infusion of hydroxyflutamide inhibited male copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking (described in Chapter 6, Part 2). The results from these experiments did not support the hypothesis that constant infusion of hydroxyflutamide would inhibit the restoration of male copulatory behavior better than daily injections. Nevertheless, these experiments showed that copulatory performance (as measured in the hit rate) was one aspect of male copulatory behavior that was both androgen- (i.e., hit rate was decreased in males treated with estradiol alone) and androgen receptor-dependent (i.e., hit rate was decreased in males treated with testosterone and hydroxyflutamide). This was in contrast to all the other aspects of male copulatory behavior (such as mount latency, ejaculatory latency, postejaculatory interval, etc.), which were regulated in a binary fashion (i.e., present or absent) rather than gradually impaired as with hit rate.

Specific Aim #4:

To assess the relative contribution of androgen receptor and estrogen receptor action on the regulation of hormone-dependent sociosexual behaviors in male rats by the combined systemic administration of an antiandrogen and an antiestrogen.

Even though blocking estrogen receptors did not inhibit testosterone's restoration of male copulatory behavior (described in Chapter 5, Part 4), estradiol still had a clear role in mediating male copulatory behavior (described in Chapter 4). In order to test if androgen receptor action contributed to estradiol's role in mediating male copulatory behavior, both hydroxyflutamide (antiandrogen) and RU 55668 (antiestrogen) were used to test if the combined administration of these two compounds would have a stronger inhibitory effect on male copulatory behavior than with either compound alone (described in Chapter 6).

The hypothesis was that cell nuclear androgen receptor action interacted with cell nuclear estrogen receptor action to mediate the effects of estradiol on male copulatory behavior, in a way that was different from when either receptor was blocked singly. However, the combined administration of both hydroxyflutamide and RU 55668 was not different from treatment with either antagonist alone.

The Role of Estrogen Receptors in Mediating Copulatory Behavior in Adult Male Rats

The concept that cell nuclear estrogen receptor action might not be necessary for the restoration of male rat copulatory behavior is actually not very new. In fact, the following statement was made in a review on this subject over two decades ago (Perez Palacios et al. 1975 p. 1003): "Since antiestrogens and antiandrogens are unable to inhibit the hormonal induced copulatory behavior, it is suggested that androgens and/or estrogens do not necessarily require the classical intracellular receptors in order to induce male behavioral response as occurs for other effects at peripheral target organs." It is interesting to note that at the time this statement was published, neither antiestrogens nor antiandrogens were capable of inhibiting the restoration of male copulatory behavior in rats. However, the use of better antiandrogens, such as hydroxyflutamide (McGinnis and Mirth 1986), which is not a partial agonist, provided a way to prove that cell nuclear androgen receptors were involved in mediating male copulatory behavior in rats. Since all the antiestrogens previously used in male rats, which did not inhibit the restoration of male copulatory behavior (such as MER-25, cis-clomiphene, and tamoxifen (Beyer et al. 1976b); and CI-628 (Whalen et al. 1972)), have been subsequently found to be partial agonists (reviewed in: Jordan 1984, Wolf and Fuqua 1995), the hypothesis that a "pure" antiestrogen (i.e., not a partial agonist, such as ICI 182780 or RU 58668) would inhibit the restoration of male rat copulatory behavior was reasonable. The logic for this hypothesis had its foundation in the antiandrogen literature, since antiandrogens, which were partial agonists (such as cyproterone acetate (Kondo et al. 1996)), were also unable to inhibit the restoration of male copulatory behavior in rats (Zucker 1966, Whalen and Edwards 1969).

The role of cell nuclear estrogen receptors in the restoration of male copulatory behavior was tested with the use of "pure" (i.e., without estrogenic activity) estrogen receptor antagonists (Van de Velde et al. 1994, Nicholson et al. 1996). Estradiol plays a

central role in the restoration of male copulatory behavior in rats, but the present studies indicate that estradiol's effects on the restoration of male copulatory behavior might not be mediated by classical cell nuclear estrogen receptors. One aspect of this work which differentiates it from previous studies with estrogen receptor antagonists in adult male rats (Sodersten and Larsson 1974, Luttge 1975, Beyer et al. 1976b), was that a separate measure of estrogen receptor action was assessed, in addition to male copulatory behavior. Cell nuclear estrogen receptor binding assays were used to determine the extent of estrogen receptor inhibition in the brain. These assays showed that brain cell nuclear estrogen receptor binding levels in testosterone-treated gonadectomized male rats treated with the antiestrogen RU 58668 were significantly decreased relative to the level found in testosterone-treated gonadectomized male rats (positive controls). Furthermore, the antiestrogen treated males had brain cell nuclear estrogen receptor binding levels that were not significantly different from gonadectomized male rats (negative controls). Therefore, this work indicates that male copulatory behavior in rats can be restored in the absence of brain cell nuclear estrogen receptor binding.

An alternative approach to address the role of estradiol in the male rat brain was tried by testing the feasibility of using a protein-steroid conjugate in restoring male copulatory behavior in male rats treated with the aromatase inhibitor fadrozole. If estradiol is acting at the cell membrane and not through classical cell nuclear estrogen receptors, administering estradiol-17β-HME-BSA (Steraloids, Inc., Wilton, NH), which reportedly cannot pass through cell membranes (Berthois et al. 1986, Ramirez et al. 1996), could be as effective as estradiol in mediating male sexual behavior. This would show that estrogen action at the cell membrane is necessary for the restoration of male sexual behavior. There is even a precedent for this hypothesis in female hamsters, where it has been shown that progesterone action at the cell membrane in the ventral tegmental area is necessary for female sexual behavior, in addition to cell nuclear progesterone receptor action in the ventromedial hypothalamus (Frye et al. 1992, DeBold and Frye 1994a). In theory, if

estradiol-17β-HME-BSA does not cross cell membranes, it should not get into cells and activate cell nuclear estrogen receptors. However, ICV administration of estradiol-17β-HME-BSA for one week (~2 μg estradiol equivalent per day) resulted in significantly elevated cell nuclear estrogen receptor binding in the brain (ANOVA F (3, 28) = 12.475, $p < 0.0001$, see Appendix A, Figure 66). This indicated that estradiol-17β-HME-BSA (or possibly free estradiol following the cleavage of the protein-steroid conjugate) is getting into cells and activating cell nuclear estrogen receptors. Therefore, using estradiol-17β-HME-BSA over the long time periods needed to study male copulatory behavior (i.e., several weeks) would not be feasible, because there would be no way to verify whether the estradiol-17β-HME-BSA was not activating cell nuclear estrogen receptors.

Many cell nuclear estrogen receptor-mediated responses have been shown to be blocked by estrogen receptor antagonists. The best example of this is female copulatory behavior in rats (Roy and Wade 1977, Wade and Blaustein 1978, Vagell and McGinnis 1996). However, in male rats, the effects of estradiol on sexually dimorphic brain development can also be blocked by antiestrogen treatment (Dohler et al. 1986, Vega Matuszczyk and Larsson 1995). Furthermore, the present work also shows that the restoration of two behaviors in adult male rats (50 kHz vocalizations and scent marking) are impaired by antiestrogen treatment (see Chapter 5, Part 4). So, at least some effects mediated by cell nuclear estrogen receptors in male rats can be blocked with antiestrogens. It is also interesting to note that the display of lordosis behavior in male rats can be blocked with the antiestrogen MER-25 (Sodersten and Larsson 1974), in spite of the fact that MER-25 is not sufficient to inhibit testosterone's restoration of male copulatory behavior in rats (Beyer et al. 1976b). Therefore, since male copulatory behavior in rats is not blocked by antiestrogen treatment, the effects of estradiol on male copulatory behavior are probably mediated by receptors other than classical cell nuclear estrogen receptors.

So, this brings up the question: If some of the effects of estradiol in the male rat brain are not mediated by classical cell nuclear estrogen receptors, then through what

mechanism is estradiol acting? Currently, there is no direct answer to this question, and it is possible that there will be several mechanisms, because there are many different nonclassical mechanisms through which estradiol could signal in the brain (reviewed in: Baulieu and Robel 1995, Brann et al. 1995). However, one likely mechanism is that estradiol modulates membrane-bound neurotransmitter receptors (reviewed in: Horvat et al. 1995, Ramirez and Zheng 1996, Joels 1997, Moss et al. 1997). For example, estradiol has been shown to rapidly uncouple μ-opioid receptors in the female rat hypothalamus (Schwarz and Pohl 1994) and $GABA_B$ receptors in female guinea pig hypothalamic slices (Lagrange et al. 1996). These rapid (< 20 min.) effects of estradiol have been postulated to occur through a nonclassical estrogen receptor that activates protein kinase A (Lagrange et al. 1997). Estradiol has also been shown to induce rapid electrophysiological effects (reversible depolarization and increased input resistance) in CA1 pyramidal neurons from male and female rats (Wong and Moss 1991). However, with the exceptions of progesterone's nongenomic role in mediating female sexual behavior in hamsters (reviewed in DeBold and Frye 1994b), and on the nongenomic effects of steroid hormones on gonadotropin release (reviewed in Wiebe 1997), there are few well established responses that are mediated by steroid hormones in a nonclassical manner. In conclusion, the present work clears the way for a new understanding of the effects of estradiol in the adult male rat brain, which is independent of cell nuclear estrogen receptors.

Models for How Androgens and Estrogens Mediate Hormone-Dependent Sociosexual Behaviors in Adult Male Rats

The following is a model for how androgens and estrogens mediate hormone-dependent sociosexual behaviors in adult male rats. It represents a conceptual framework for the actions of steroid hormones and their receptors in the brain. This model does not go into detail concerning neuroanatomical localization (such as Wood (1997) on male hamster sexual behavior), because there is not enough data on the roles of different steroid receptors in mediating these hormone-dependent sociosexual behaviors, within individual brain regions, to formulate such a model. Moreover, relatively little is known about which neural sites are involved in mediating partner preference, 50 kHz vocalizations, and scent marking in adult male rats. The foundation for this model is that the actions of steroid hormones in the brain are mediated by selective receptors located at key regulatory points in neural circuits, which mediate sociosexual behaviors in adult male rats. If steroid hormones are functioning like "keys" through the activation of steroid receptors (acting as switches) at certain regulatory points within neural circuits, there are a limited number of ways in which these neural circuits could be wired (displayed in Figure 48) to support the hormonal regulation of sociosexual behaviors.

The presence of cues (olfactory, visual, auditory, tactile) from sexually receptive females are capable of stimulating sociosexual behaviors in adult male rats under certain conditions (reviewed in Stern 1990). One condition that is sufficient for the presentation of sociosexual behaviors in adult male rats is the presence of testosterone in the blood-stream. In the absence of the appropriate steroid hormones (for example, in a gonadectomized male rat), the expression of hormone-dependent sociosexual behaviors is generally inhibited, even in the presence of the proper environmental cues (such as the ones displayed by sexually receptive female rats). Treatment with testosterone restores these hormone-dependent sociosexual behaviors over the course of two weeks in the adult male rats

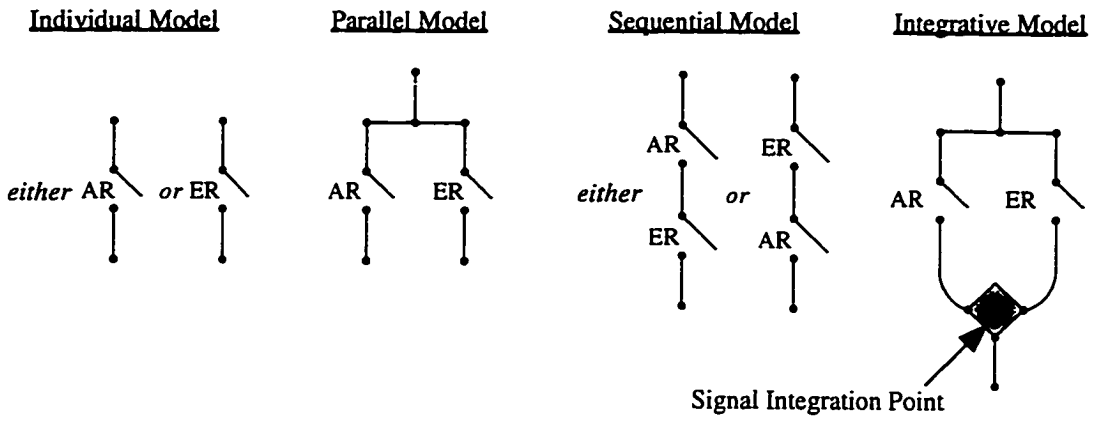


Figure 48. Various Models for How Multiple Steroid Receptors Could Play Regulatory Roles in Neural Circuits

Abbreviations: = AR = any androgen receptor, ER = any estrogen receptor

studied in the present work. Therefore, testosterone, or possibly a testosterone metabolite (such as estradiol), can function to regulate these behaviors in a hormone-dependent manner. The environmental cues from sexually receptive female rats, for eliciting hormone-dependent sociosexual behaviors in adult male rats, serve as the input in the models described hereafter.

In the Individual Model shown in Figure 48, either androgen receptors or estrogen receptors could separately act as regulators in a neural circuit. The best example of a behavior which fits the Individual Model is partner preference, because this behavior is exclusively regulated by cell nuclear androgen receptors. Blocking either aromatase with fadrozole or cell nuclear estrogen receptors with RU 58668 has no affect on the restoration of partner preference. Moreover, treatment of gonadectomized male rats with estradiol alone is insufficient to restore partner preference. Therefore, estradiol is not involved in the restoration of partner preference.

The other three models shown in Figure 48 involve the action of both androgen and estrogen receptors. Male copulatory behavior in rats is an example of a behavior that is regulated by the actions of both testosterone and estradiol. If the formation of estradiol is blocked with an aromatase inhibitor, male copulatory behavior cannot be restored.

Moreover, estradiol alone is insufficient for the restoration of male copulatory behavior. So, the action of both androgens and estrogens are necessary for the restoration of male copulatory behavior in rats.

In the Parallel Model, either androgen receptor activation or estrogen receptor activation would be sufficient for the restoration of the behavior. This model predicts that if only one of the receptors was inhibited, the animal would still behave because the signal would then be passed on by the other receptor in the parallel circuit. However, none of the sociosexual behaviors studied in this work fit this model, because it predicts that both androgen and estrogen receptors would both need to be blocked at the same time in order to inhibit the restoration of the behavior, and blocking androgen receptors alone was sufficient to inhibit the restoration of all the behaviors studied in this work.

The Sequential Model does fit the data, because blocking one of the receptors in the hypothetical chain would be sufficient to inhibit the behavior. For example, it would fit male copulatory behavior because blocking cell nuclear androgen receptors is sufficient to inhibit the restoration of male copulatory behavior. However, there are some shortcomings in the Sequential Model. First, there is the problem of order in the pathway. Which receptor would be first in the chain? With behavioral data alone, it is difficult to order this pathway. Second, the sequential model does not provide for the possibility that the activation of the upstream receptor could stimulate the behavior under certain conditions. For example, studies have shown that male copulatory behavior in rats can be restored if enough dihydrotestosterone (a nonaromatizable androgen, reviewed in Yahr 1979) or estradiol (reviewed in Sodersten 1979) are given alone. Therefore, it would not be possible for estradiol to activate male copulatory behavior in the absence of androgens, if estrogen receptors were upstream of androgen receptors. So, the Sequential Model does not fully support the experimental data.

The only model in Figure 48 that supports the available data is the Integrative Model. This model is actually an extension of the Parallel Model, except for the fact that the

signals from each receptor are linked together at a third point (conceptually) where the signals from each receptor converge. The idea here is that both signals are necessary to activate the rest of the neural circuit mediating the behavior. The Integrative Model supports the data showing that inhibition of cell nuclear androgen receptor action alone is sufficient to block the restoration of male copulatory behavior. Furthermore, it allows for the possibility that enough stimulation on one side of the pathway could be sufficient to activate the behavior. For example, treating male rats with large amounts of estradiol has been shown to restore of male copulatory behavior (Sodersten 1973), even though lower doses of estradiol do not restore of male copulatory behavior (McGinnis and Dreifuss 1989). Moreover, male rats implanted peripherally with subcutaneous dihydrotestosterone capsules do not ordinarily copulate, but male rats that are implanted with dihydrotestosterone-filled cannulas into the medial preoptic area along with subcutaneous dihydrotestosterone capsules will copulate (Butera and Czaja 1989). This indicates that even though both androgens and estrogens are needed in the brain for the restoration of male copulatory behavior, enough stimulation with either hormone can be sufficient for the restoration of male copulatory behavior.

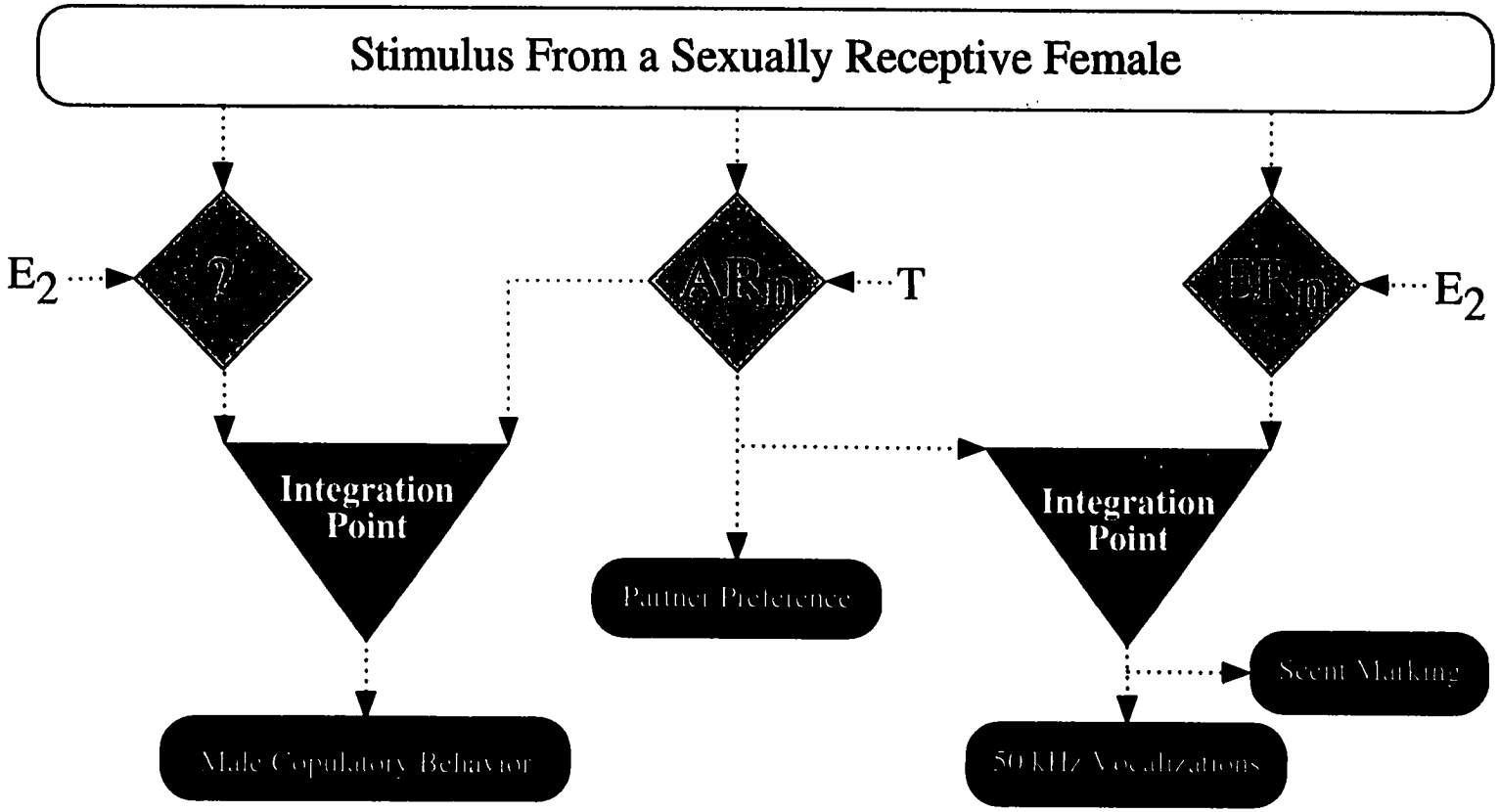
Application of the Integrative Model for the Hormonal Regulation of Sociosexual Behaviors in Adult Male Rats

Throughout most of the present work, four different hormone-dependent sociosexual behaviors were studied (male copulatory behavior, partner preference, 50 kHz vocalizations, and scent marking). Two fundamental aspects relating these behaviors together is that they decline after gonadectomy, and that they can be restored with implanting testosterone capsules in adult male rats. However, these behaviors are not all restored by the actions of testosterone is the same manner. For example, male copulatory behavior is dependent on the aromatization of testosterone to estradiol, while the restoration

of partner preference is not. The following model places these four behaviors together in terms of how the actions of multiple steroid hormone receptors could serve to regulate these behaviors (see Figure 49).

The least complex behavior that was studied, with respect to the number of hormones involved, was partner preference. Neither, the aromatase inhibitor or the antiestrogen blocked the restoration of partner preference. Moreover, estradiol alone was insufficient to restore partner preference in the absence of testosterone. However, blocking cell nuclear androgen receptors blocked the restoration of partner preference. Therefore, the model depicts partner preference directly after the action of cell nuclear androgen receptors, without the influence of estrogen receptor action.

The three other sociosexual behaviors studied (male copulatory behavior, 50 kHz vocalizations, and scent marking) were modulated by the effects of both androgen and estrogen receptor action. However, while estradiol was necessary for the restoration of male copulatory behavior in adult male rats, cell nuclear estrogen receptor action was not (discussed in Chapter 5, Part 4). This indicated that estrogen receptors other than the classical cell nuclear estrogen receptors were involved in mediating male copulatory behavior. Therefore, there is another receptor, or receptors, which mediates the effects of estradiol in adult male rats, in addition to classical cell nuclear estrogen receptors. On the other hand, the restoration of 50 kHz vocalizations and scent marking were impaired by antiestrogen treatment. This indicated that cell nuclear estrogen receptors were involved in the restoration of these two behaviors. Consequently, the model in Figure 49 depicts two different receptors for estradiol, one marked with a question mark for the unknown estrogen receptors and the other marked ER_n for classical cell nuclear estrogen receptors. Two different "integration points" are shown in the model because different estrogen receptors appear to mediate male copulatory behavior and 50 kHz vocalizations/scent marking, in spite of the fact that the restoration of all three of these behaviors can be inhibited with antiandrogen treatment.



Abbreviations:
E_2 = estradiol
T = testosterone
? = unknown receptor(s) for estradiol
AR_n = cell nuclear androgen receptors
ER_n = cell nuclear estrogen receptors

Note: The triangles labeled "integration point" represent theoretical sites where androgenic and estrogenic signals converge in the processing of hormone-dependent behavioral responses. However, this model does not necessarily imply that multiple sites are involved. In fact, this kind of signal integration could even occur within a single cell in a neural circuit.

Figure 49. Integrative Model for the Hormonal Regulation of Sociosexual Behaviors in Adult Male Rats

Future Studies

Cell Nuclear Estrogen Receptor Gene Inhibition Studies

Until the development of more selective and potent antiestrogens, there is not much left to do with existing antiestrogens (such as tamoxifen, ICI 182780, or RU 58668) in the study of copulatory behavior in adult male rats. However, the role of cell nuclear estrogen receptors in mediating 50 kHz vocalizations in adult male rats is one area where future studies with antiestrogens would be useful. Specifically, intracranial implant studies (similar to McGinnis et al. (1996)) with the antiestrogen RU 58668 would aid in determining the site(s) of cell nuclear estrogen receptor action mediating the restoration of 50 kHz vocalizations in adult male rats. The results of intracranial implant studies with the antiestrogen RU 58668 could then be compared with the results from intracranial implant studies with the antiandrogen hydroxyflutamide to determine if similar brain regions are involved in mediating the effects of cell nuclear androgen and estrogen receptors in the restoration of 50 kHz vocalizations.

To finally resolve the issue of what the cell nuclear estrogen receptor's role is in mediating male rat copulatory behavior, new experimental approaches are needed. Traditionally, the roles of receptors in different biological processes have been assessed with the use of pharmacological antagonists (i.e., drugs that block receptors; for example, using the antiandrogen hydroxyflutamide to asses the role of cell nuclear androgen receptors in mediating male copulatory behavior (McGinnis and Mirth 1986)). However, there are now tools available to side-step blocking receptors with antagonists, which involve depleting the receptors in vivo. This approach would involve using genetic engineering technology to disable the cell nuclear estrogen receptors in a controlled manner in adult male rats.

One method, which would use a similar experimental design as the estrogen receptor antagonist experiments discussed in the present chapters, is the use of antisense oligodeoxynucleotides to deplete cell nuclear estrogen receptors in specific brain regions (Szklarczyk and Kaczmarek 1995, Ogawa and Pfaff 1996). Antisense oligodeoxynucleotides can be designed to target specific complimentary mRNA sequences and inhibit the expression of genes through several possible mechanisms, such as RNase H mediated destruction of the mRNA or inhibition of the mRNA's translation start site (reviewed in: Knorre and Vlassov 1990, Persaud and Jones 1994, Herdewijn 1996). In fact, antisense oligodeoxynucleotides have been used to test the hypothesis that cell nuclear estrogen receptor action is involved in sexual differentiation of the rat brain (McCarthy et al. 1993). McCarthy et al.'s 1993 study showed that antisense oligodeoxynucleotides complimentary to the mRNA coding for the estrogen receptor-alpha (injected into the hypothalamus) protected postnatal female rat pups from the androgenizing effects of testosterone (Gorski 1993).

Unfortunately, there are a number of problems with using antisense oligodeoxynucleotides to inhibit gene expression. First, there is the question of toxicity. One strategy to prevent the degradation of antisense oligodeoxynucleotides in vivo has been the substitution of the phosphodiester backbone for a phosphorothioate backbone, but this modification has been shown to be toxic in a number of cases (Chiasson et al. 1994, Corrias et al. 1997). Second, antisense oligodeoxynucleotides complementary to the 5'-end of estrogen receptor-alpha mRNA have been shown to paradoxically upregulate the levels of estrogen receptor protein in MCF-7 cells (Santagati et al. 1997). Therefore, the use of antisense oligodeoxynucleotides to study the role of estrogen receptor action in male rat copulatory behavior has a number of pitfalls that still need to be worked out.

Another genetic engineering approach to address the question of cell nuclear estrogen receptor action in mediating male copulatory behavior would be to create strains of rats with inducible (also known as conditional) knockouts (Passman and Fishman 1994) in

both the estrogen receptor-alpha and estrogen receptor-beta genes. The key aspect of this approach would be the use of inducible, rather than constitutive, knockouts. This would be necessary to ensure that the organizational aspects of estrogen receptor action during development could be separated from their activational role in adulthood (Kawata 1995). Mice with constitutive disruptions in the gene for the estrogen receptor-alpha are currently available (Korach et al. 1996). However, since this gene is disabled throughout the animal's life, and the role of estrogen receptor-alpha is known to play a central role in the sexual differentiation of the brain, the behavior of these animals in adulthood cannot be compared with animals that have undergone wild-type development.

Unfortunately, creating inducible knockouts in rats has never been performed before. Furthermore, there are significant differences regarding reproductive behavior between rats and mice, so that gene disruption studies in mice are not directly applicable to rats. In fact, this technology has only been utilized a few times in mice (Passman and Fishman 1994, Hennighausen et al. 1995, St Onge et al. 1996, Nebert and Duffy 1997). Therefore, it is unlikely that inducible knockouts will be available for use in rats in the near future. So, until significant technical advances are made, cell nuclear estrogen receptor gene disruption studies in rats will not be feasible.

Other Future Studies

Besides the issue of what the cell nuclear estrogen receptor's role is in mediating different hormone-dependent sociosexual behaviors, there are many other pertinent questions that relate to how steroid hormones mediate different hormone-dependent sociosexual behaviors in rats remaining to study. For example, there is the question of localization: Where in the adult male rat brain are the effects of androgens and estrogens occurring that mediate the restoration of hormone-dependent sociosexual behaviors? The approaches used in the studies described in the previous chapters were specifically

designed to avoid this question, because it was necessary to first determine which behaviors were affected by the different inhibitors acting on the whole brain. This was necessary because there would be no way of knowing whether the outcome of infusing an inhibitor at one brain site was the result of that site not playing a role in mediating the behavior. For instance, it would be unlikely that intracranial implants of the antiestrogen RU 58668 would block the restoration of male copulatory behavior, since systemic injections of RU 58668 did not (along with the fact that RU 58668 significantly reduced the level of brain cell nuclear estrogen receptor binding). However, now that it is known which inhibitors block the restoration of several hormone-dependent sociosexual behaviors, localization studies using intracranial implants with the different inhibitors (such as fadrozole, RU 58668, and hydroxyflutamide) would be useful to isolate which brain regions mediate the actions of androgens and estrogens in the restoration of hormone-dependent sociosexual behaviors. In fact, intracranial implant studies with hydroxyflutamide, similar to the ones just described, have shown that androgen receptors in the ventromedial nucleus of the hypothalamus are necessary for the restoration of male copulatory behavior (McGinnis et al. 1996) and partner preference (Marilyn McGinnis, unpublished observations) in adult male rats.

Lastly, another direction for this research would be to examine the role of steroid hormone receptors in mediating other hormone-dependent behaviors than the ones investigated in this work (especially different aspects of agonistic behavior in adult male rats). The study other hormone-dependent behaviors would be tremendously useful in order to expand the search for generalized aspects of how steroid hormones mediate behavior in adult male rats. Much of the literature on the study of agonistic behavior in male rat has focused on the role of androgens, but there is also evidence that estradiol can mediate agonistic behavior (reviewed in: Knol and Egberink-Alink 1989, Albert et al. 1992). However, I am not aware of any studies, which have used recently developed antiestrogens or antiandrogens, that have examined the roles of cell nuclear androgen and

estrogen receptor activation in mediating other hormone-dependent behaviors in adult male rats.

Answers to Questions Presented in the First Chapter

1. Is estradiol (produced from the aromatization of testosterone) necessary for the restoration of male copulatory behavior and partner preference? Yes, without estradiol, derived from the aromatization of testosterone, the restoration of male copulatory behavior in adult male rats is inhibited.

2. Are male copulatory behavior and partner preference differentially regulated by testosterone and/or estradiol? Yes, in adult male rats, copulatory behavior is mediated by both androgens and estrogens, but partner preference is only mediated by androgens.

3. What are the relative contributions of testosterone and estradiol in mediating male copulatory behavior and partner preference? The model outlined in Figure 49 provides a more detailed answer to this question from the standpoint of the receptors for testosterone and estradiol. However, with regard to the steroid hormones themselves, it appears that estradiol plays a central role in the initiation of male copulatory behavior in adult male rats. The reason is that if aromatization is blocked, the majority of the animals will not display any copulatory behavior at all. If a gonadectomized male is only implanted with an estradiol capsule, delivering a physiologically relevant dose of estradiol, most males will not copulate, and those estradiol-treated males that do copulate, show decreases in copulatory performance. So, estradiol can partially activate male copulatory behavior in a small percentage of adult male rats, but the copulatory behavior in these animals is impaired without testosterone. One explanation for the small percentage of males copulating when either aromatase is blocked or the males are only treated with estradiol, is that there are a few rats with a higher sensitivity to the effects of estradiol than the males that do not copulate. Therefore, this indicates that both testosterone and estradiol are necessary for the restoration of male copulatory behavior in adult male

rats, but that without estradiol the males do not attempt to copulate. In the absence of androgenic stimulation, males attempt to copulate, but they do so inefficiently.

With regard to partner preference, no evidence was found indicating that estradiol plays a role in restoring partner preference in adult male rats. First, blocking aromatase did not inhibit the restoration of partner preference. Second, estradiol alone was insufficient to restore partner preference. Third, treatment with an antiestrogen had no effect on the restoration of partner preference.

4. Are cell nuclear androgen receptor and estrogen receptor action necessary for the restoration of different hormone-dependent sociosexual behaviors (including male copulatory behavior, partner preference, scent marking, and 50 kHz ultrasonic vocalizations)? It depends on the behavior in question. For male copulatory behavior, cell nuclear androgen receptors are necessary, but cell nuclear estrogen receptors are not. Therefore, something besides cell nuclear estrogen receptors are mediating the effects of estradiol in the brains of adult male rats. Partner preference is mediated only by the effects of cell nuclear androgen receptors. Estradiol or cell nuclear estrogen receptors do not appear to be involved in mediating partner preference. On the other hand, 50 kHz vocalizations and scent marking appear to be mediated by both cell nuclear estrogen receptors and cell nuclear androgen receptors. However, blocking both cell nuclear estrogen receptors and cell nuclear androgen receptors at the same time did not result in a synergistic inhibition of any behavior studied.

5. Does cell nuclear androgen receptor action contribute to the role of cell nuclear estrogen receptor action in the restoration of sociosexual behaviors? No, there were no additive or synergistic effects on any of the sociosexual behaviors measured resulting from the combined administration of the antiestrogen RU 58668 and the antiandrogen hydroxyflutamide.

Appendix A - Supplementary Figures and Tables

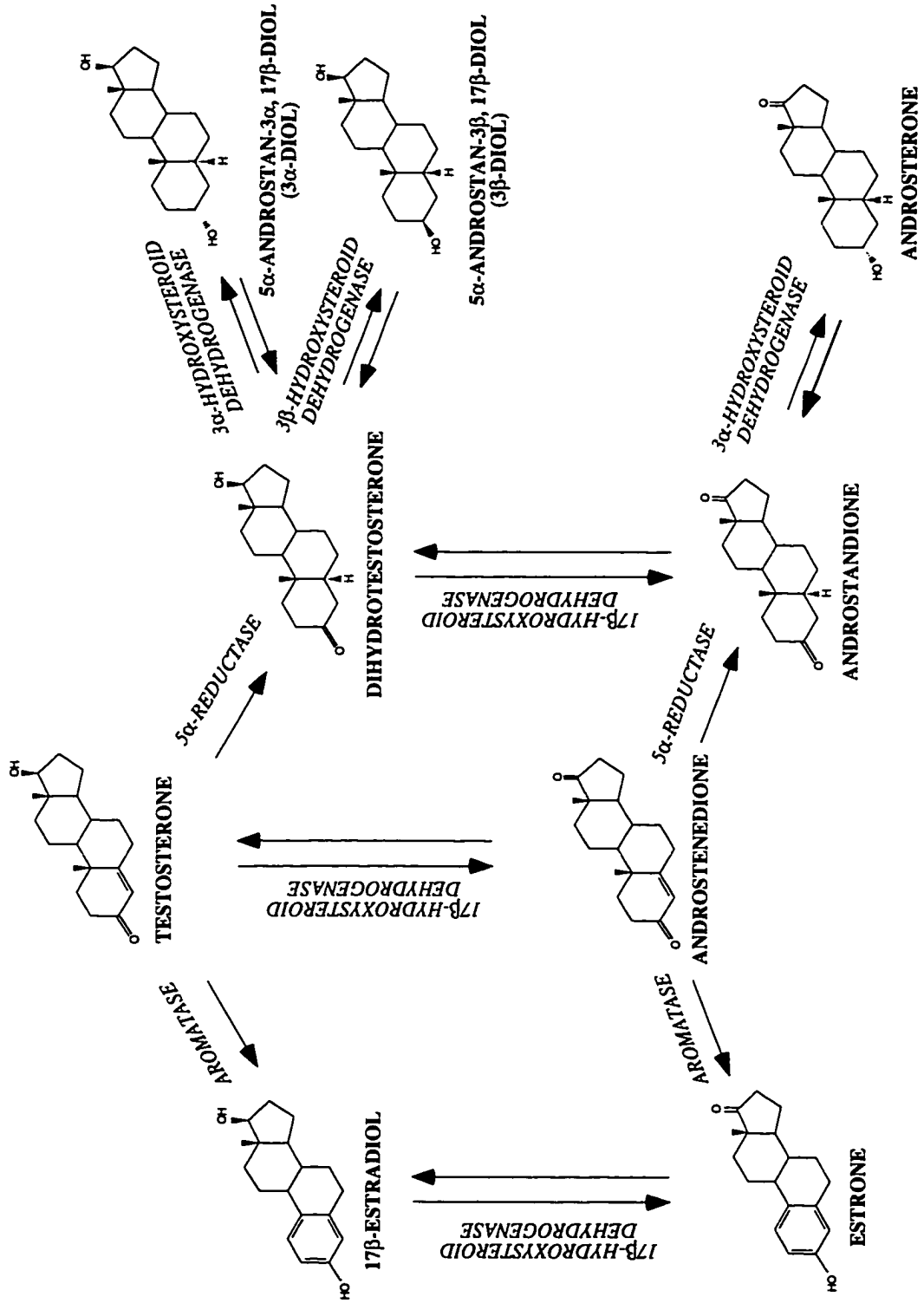
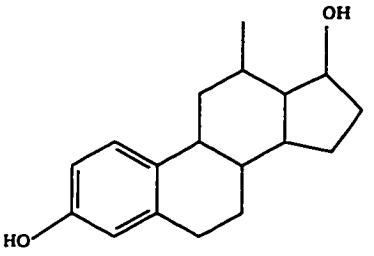
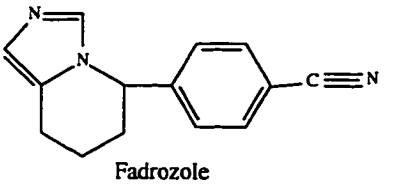


Figure 50. Metabolic Relationships of Testosterone's Metabolites



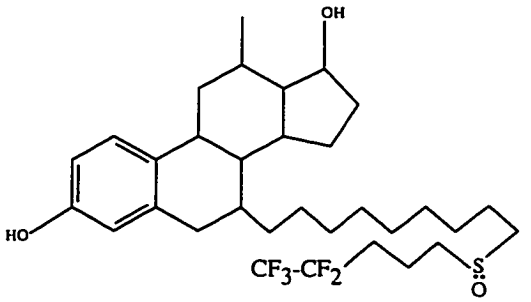
Estradiol

Aromatase Inhibitor



Fadrozole

Antiestrogens



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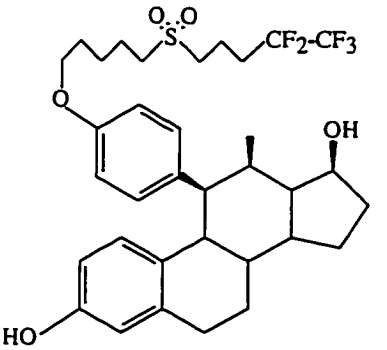
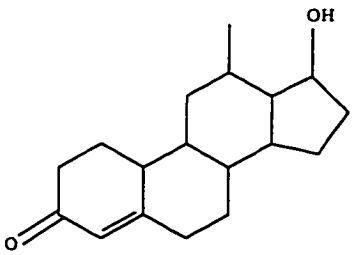
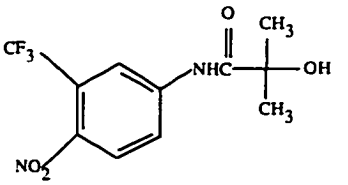


RU 58668



Testosterone

Antiandrogen



Hydroxyflutamide
(SCH 16423)

Figure 51. Inhibitor Structures

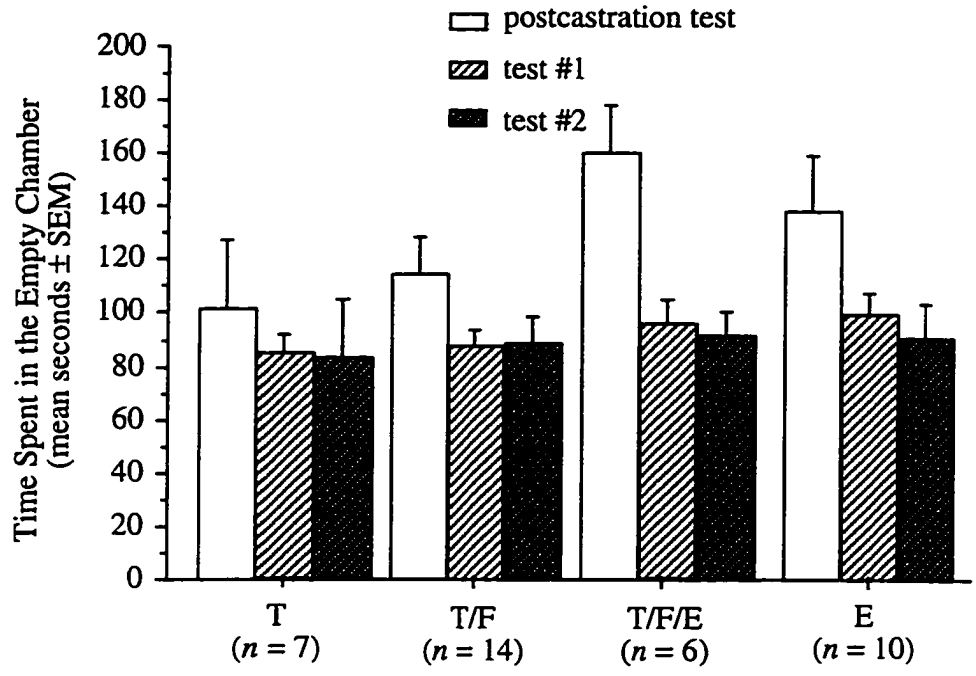


Figure 52. Time Spent in the Empty Chamber - Fadrozole Experiment

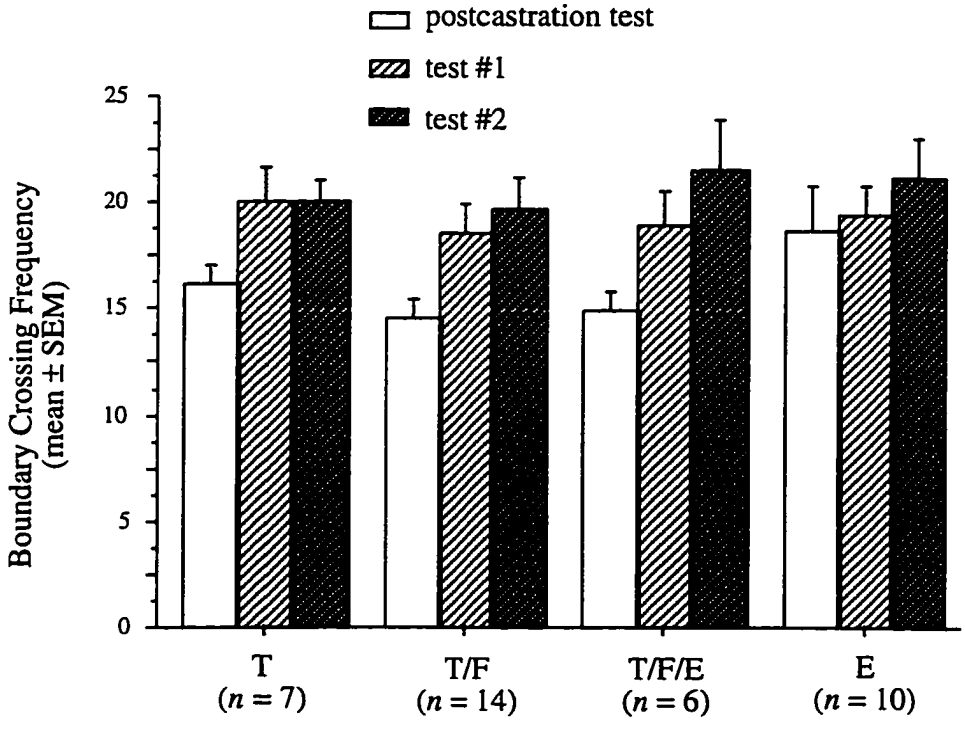


Figure 53. Boundary Crossing Frequency - Fadrozole Experiment

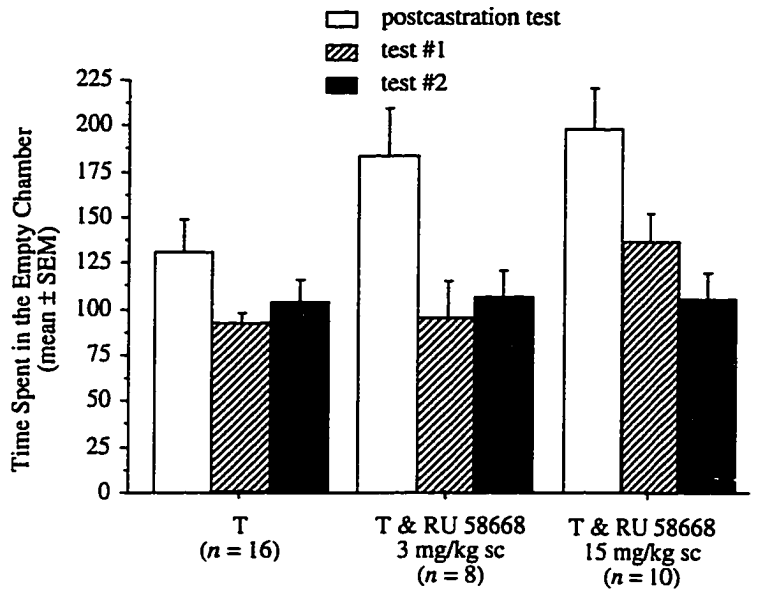


Figure 54. Effect of RU 58668 on Time Spent in the Empty Chamber

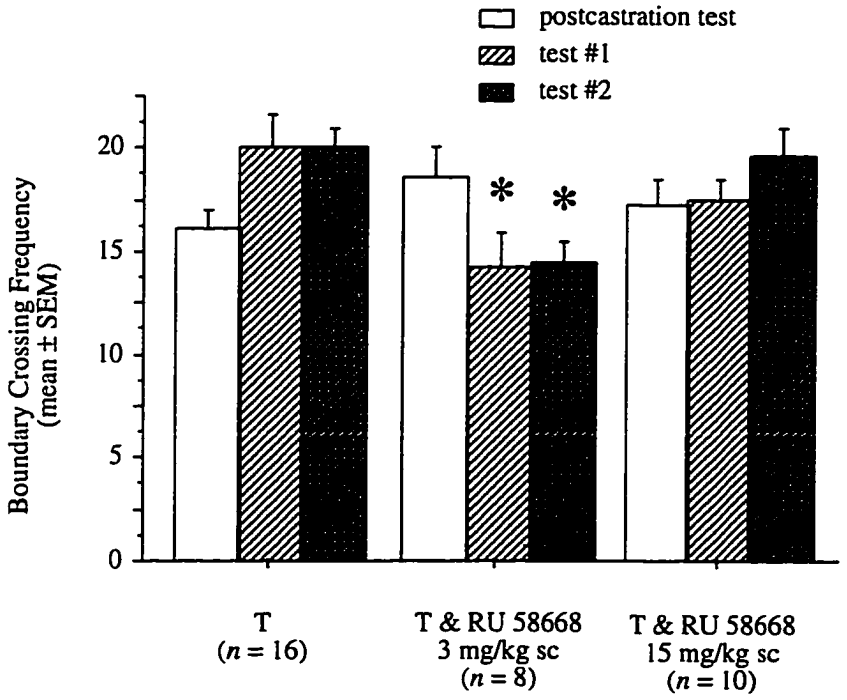


Figure 55. Effect of RU 58668 on Boundary Crossing Frequency

Note: Daily injections of 3 mg/kg sc RU 58668 reduced testosterone's restoration of boundary crossing frequency. Asterisks indicate $p < 0.05$ vs. the T group for the corresponding test. However, this effect was not evident in the T & 15 mg/kg sc RU 58668 group.

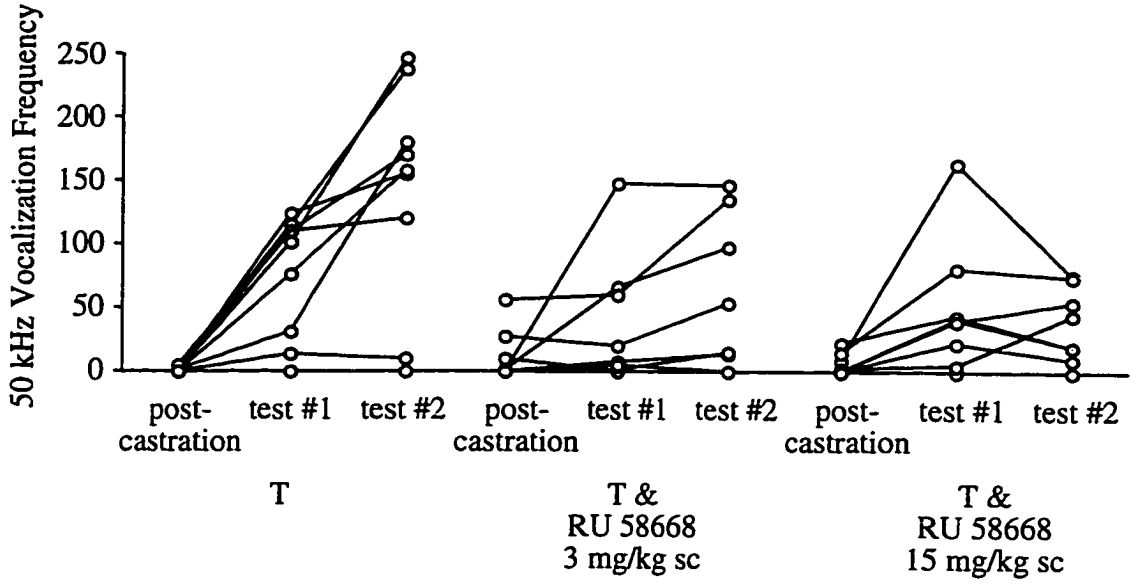


Figure 56. Effect of RU 58668 on the Restoration of 50 kHz Vocalizations - Individual Line Plots

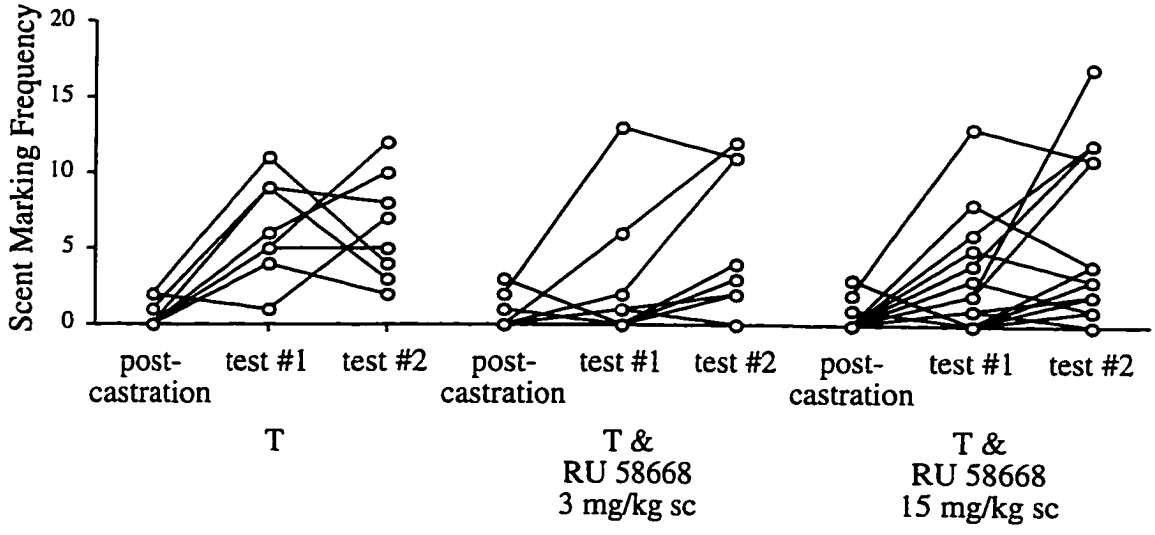


Figure 57. Effect of RU 58668 on the Restoration of Scent Marking - Individual Line Plots

Note: Each line connects each animal's scores across the three tests. The purpose of this figure is to graphically depict the data summarized in the paired difference scores.

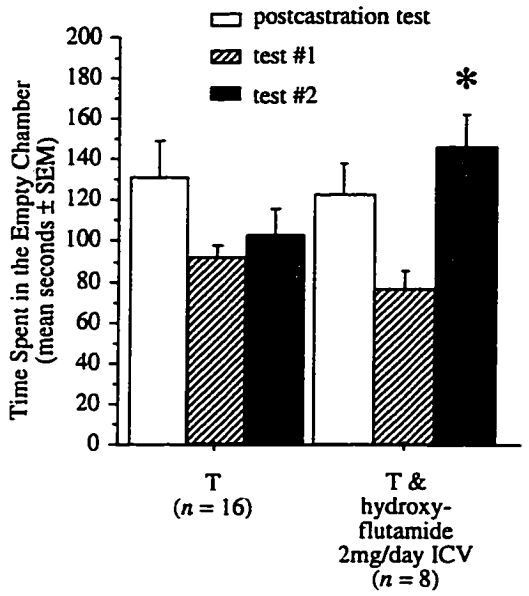


Figure 58. Effect of ICV Delivery of Hydroxyflutamide on Time Spent in the Empty Chamber

Note: Asterisks indicate $p < 0.05$ vs. the T group for the corresponding test.

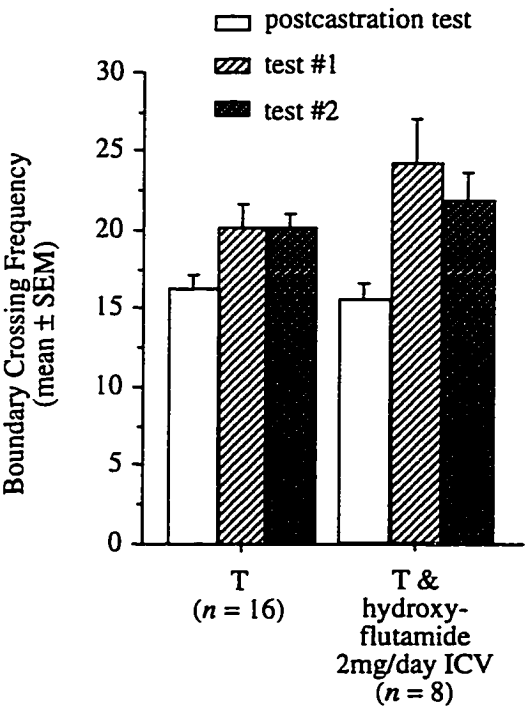


Figure 59. The Effect of ICV Delivery of Hydroxyflutamide on Boundary Crossing Frequency

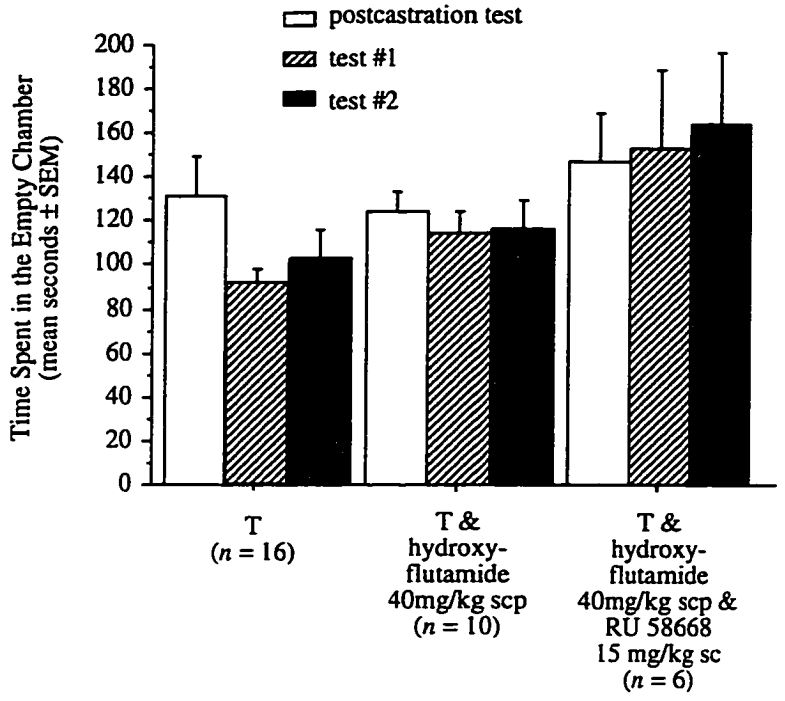


Figure 60. Effect of Hydroxyflutamide and/or RU 58668 on Time Spent in the Empty Chamber

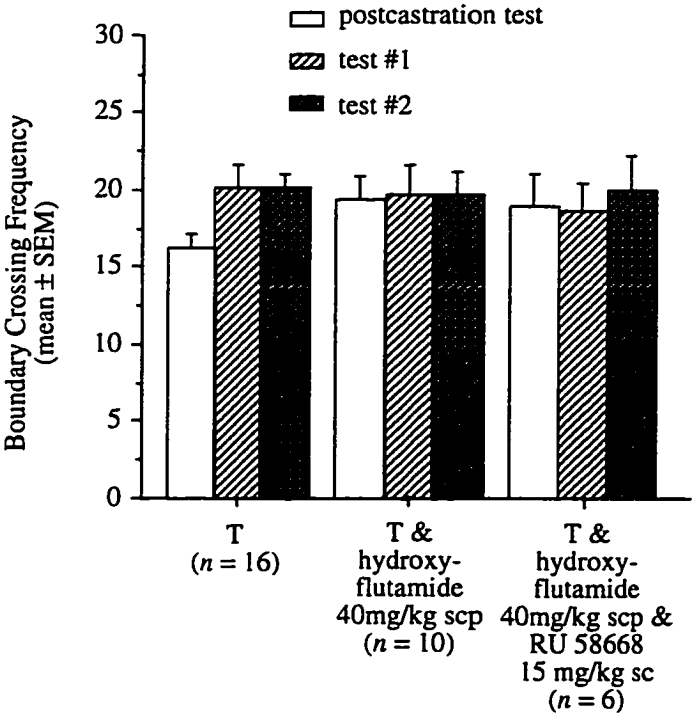


Figure 61. Effect of Hydroxyflutamide and/or RU 58668 on Boundary Crossing Frequency

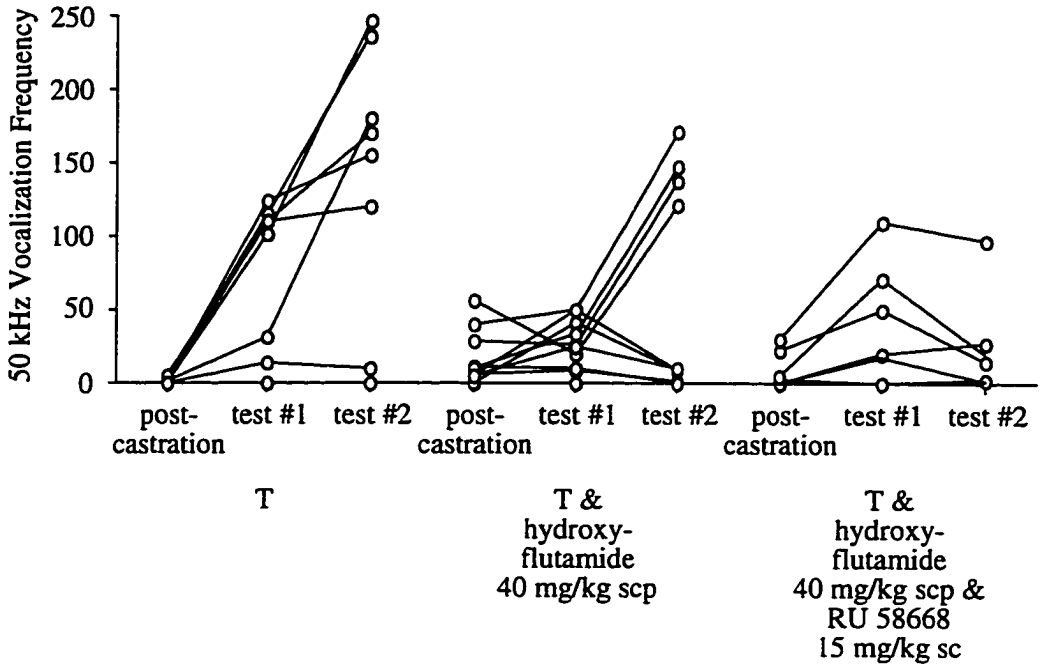


Figure 62. Effects of Hydroxyflutamide and RU 58668 on the Restoration of 50 kHz Vocalizations - Individual Line Plots

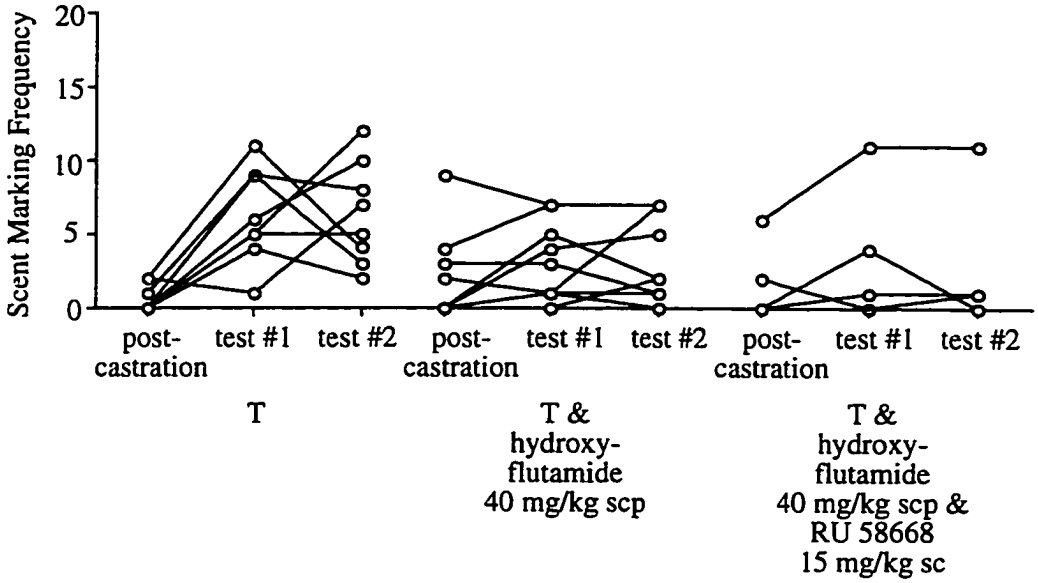


Figure 63. Effects of Hydroxyflutamide and RU 58668 on the Restoration of Scent Marking - Individual Line Plots

Note: Each line connects each animal's scores across the three tests. The purpose of these figures is to graphically depict the data summarized in the paired difference scores. The abbreviations sc and scp refer to daily subcutaneous injections and subcutaneous mini-osmotic pumps, respectively.

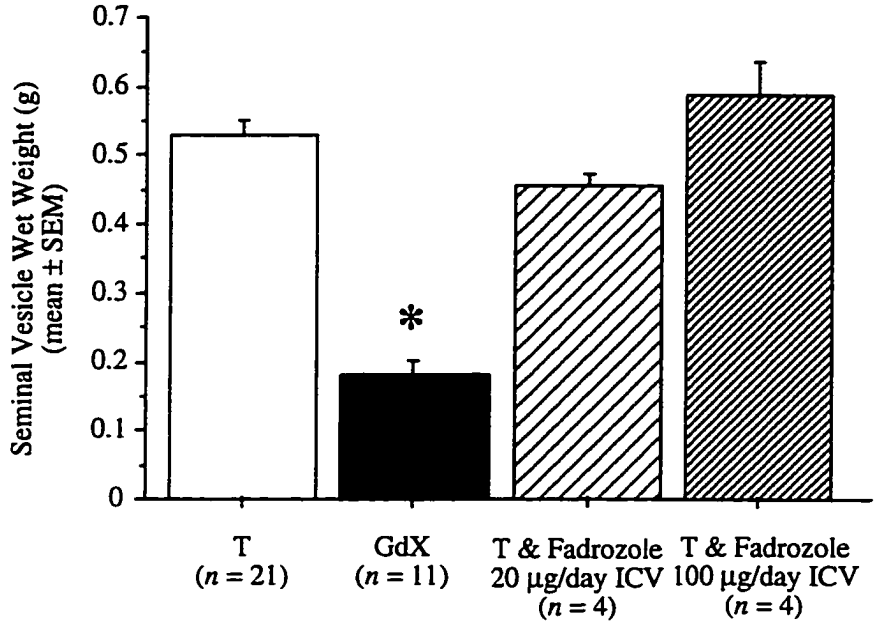


Figure 64. Effect of Fadrozole on Seminal Vesicle Weights

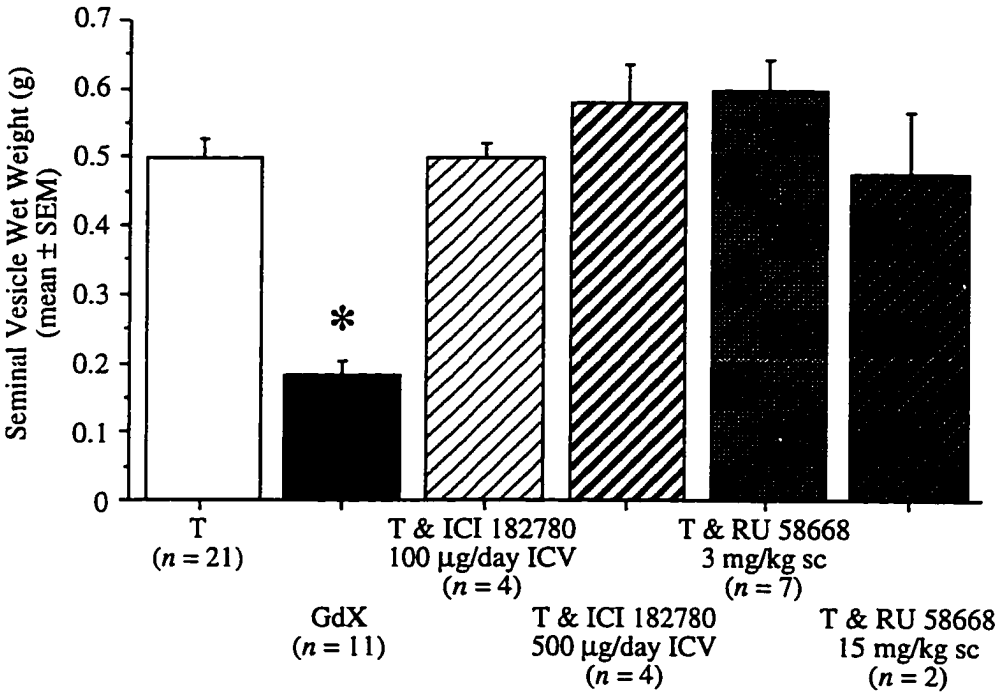


Figure 65. Effects of the Antiestrogens ICI 182780 and RU 58668 on Seminal Vesicle Weights

Note: Asterisks indicate $p < 0.05$ vs. T, Fisher's PLSD post hoc test.

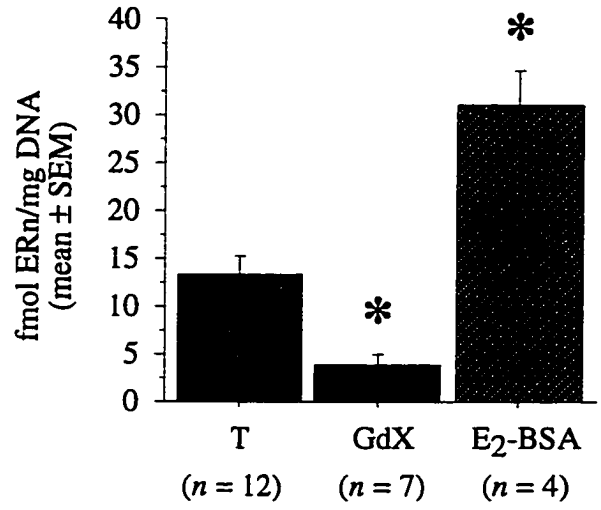


Figure 66. Effect of Estradiol-17β-HME-BSA on Cell Nuclear Estrogen Receptor Binding in the Brain

Brain (pooled hypothalamus, preoptic area, amygdala, and septum) cell nuclear estrogen receptor binding was measured after one week of ICV administration (2 µg/day) of estradiol-17β-HME-BSA (E_2-BSA) in gonadectomized males implanted subcutaneously with two, 10 mm Silastic capsules filled with testosterone. Gonadectomized males in the T group were only implanted with testosterone capsules, and the GdX males were did not receive either treatment.
* $p < 0.05$ vs. T, Fisher's PLSD post hoc test

Table 16. Male Copulatory Behavior - Fadrozole Pilot Study

	two, 10 mm testosterone capsules			
		fadrozole (μg/day ICV)		
		1	20	100
number of animals tested	(11)	(6)	(4)	(4)
Mount Frequency $F(1,15) = 0.041, p = 0.8430$	8 ± 5	8 ± 1	*	*
Intromission Frequency $F(1,15) = 1.585, p = 0.2273$	14 ± 4	12 ± 1	*	*
Mount Latency $F(1,15) = 1.119, p = 0.3069$	51 ± 64	118 ± 79	*	*
Intromission Latency $F(1,15) = 1.280, p = 0.2757$	89 ± 92	167 ± 80	*	*
Ejaculation Latency $F(1,15) = 0.492, p = 0.4938$	432 ± 205	508 ± 94	*	*
Post Ejaculatory Interval $F(1,15) = 1.311, p = 0.2702$	339 ± 53	310 ± 19	*	*
Hit Rate $F(1,15) = 0.260, p = 0.6176$	65 ± 17	61 ± 3	*	*
Intercopulatory Interval $F(1,15) = 0.409, p = 0.5320$	24 ± 8	27 ± 5	*	*
Copulatory Duration $F(1,15) = 0.0003, p = 0.9866$	342 ± 144	341 ± 85	*	*

Note. Data shown are means ± standard errors. Asterisks indicate animals who did not display any copulatory behavior. Administration of 1 μg/day fadrozole ICV did not have any significant effect on male copulatory behavior. The number of animals tested equaled the number of animals who displayed male copulatory behavior (*n*). Only one behavioral test was given 13 days after implant surgery. One-way ANOVA statistics are listed below the parameter names.

Table 17. Effects of Fadrozole on Male Copulatory Behavior

		two, 10 mm testosterone capsules			
			20 µg fadrozole/day ICV		
	Test			1, 5 mm 1% estradiol capsule	
number of animals tested		(16)	(14)	(6)	(10)	
Mount Frequency						
F (3,19) = 13.6, $p < 0.0001$	1	8 ± 1 (12)	4 ± 2 (3)	4 ± 1 (5)	23 ± 3 (3)	*
F (3,22) = 14.8, $p < 0.0001$	2	5 ± 1 (16)	9 ± 2 (2)	5 ± 1 (6)	20 ± 5 (2)	*
Intromission Frequency						
F (3,18) = 2.0, $p = 0.1412$	1	14 ± 2 (12)	6 ± 1 (3)	16 ± 2 (5)	14 ± 1 (2)	
F (3,23) = 1.2, $p = 0.3209$	2	10 ± 1 (16)	11 ± 3 (2)	13 ± 2 (6)	16 ± 1 (2)	
Mount Latency						
F (3,19) = 1.6, $p = 0.2219$	1	71 ± 40 (12)	268 ± 241 (3)	19 ± 6 (5)	4 ± 2 (3)	
F (3,23) = 0.2, $p = 0.8745$	2	69 ± 30 (16)	99 ± 73 (2)	41 ± 28 (6)	47 ± 37 (2)	
Intromission Latency						
F (3,18) = 1.7, $p = 0.2088$	1	81 ± 41 (12)	280 ± 242 (3)	28 ± 12 (5)	307 ± 287 (2)	
F (3,23) = 2.1, $p = 0.1231$	2	84 ± 35 (16)	103 ± 73 (2)	45 ± 29 (6)	367 ± 347 (2)	
Ejaculation Latency						
F (3,18) = 1.2, $p = 0.3318$	1	450 ± 92 (12)	472 ± 364 (3)	271 ± 75 (5)	809 ± 140 (2)	
F (3,22) = 5.7, $p = 0.0048$	2	277 ± 51 (16)	261 ± 153 (2)	249 ± 54 (6)	1022 ± 539 (2)	*
Post Ejaculatory Interval						
F (3,17) = 1.2, $p = 0.3435$	1	362 ± 33 (12)	498 ± 65 (3)	400 ± 21 (5)	401 ± 23 (2)	
F (3,22) = 2.2, $p = 0.1235$	2	342 ± 14 (16)	209 ± 209 (2)	385 ± 29 (6)	364 ± 7 (2)	
Hit Rate						
F (3,18) = 12.0, $p = 0.0002$	1	64 ± 3 (12)	62 ± 7 (3)	82 ± 4 (5)	36 ± 5 (2)	*
F (3,22) = 2.5, $p = 0.0842$	2	69 ± 3 (16)	60 ± 10 (2)	73 ± 5 (6)	45 ± 5 (2)	
Intercopulatory Interval						
F (3,18) = 1.7, $p = 0.2056$	1	24 ± 3 (12)	25 ± 12 (3)	15 ± 2 (5)	37 ± 10 (2)	
F (3,22) = 8.1, $p = 0.0008$	2	18 ± 1 (16)	18 ± 7 (2)	16 ± 3 (6)	42 ± 11 (2)	*
Copulatory Duration						
F (3,18) = 0.8, $p = 0.5161$	1	369 ± 91 (12)	192 ± 123 (3)	243 ± 64 (5)	502 ± 147 (2)	
F (3,22) = 8.2, $p = 0.0008$	2	194 ± 28 (16)	232 ± 163 (2)	205 ± 35 (6)	655 ± 192 (2)	*

Note. Data shown are means ± standard errors. The numbers in parentheses below the standard errors are the number of animals displaying the behavior (n). Test 1 was 7 days after implant surgery, and Test 2 was 13 days after implant surgery. One-way ANOVA statistics for each test are listed below the parameter names. Repeated Measures ANOVAs were not performed because a pretest was not done, and thus, baseline data was not available. Asterisks indicate $p < 0.05$ vs. the testosterone group, Fisher's PLSD Post-hoc Test.

Table 18. Effects of ICI 182780 on Male Copulatory Behavior

	two, 10 mm testosterone capsules			
		ICI 182780 (μg/day ICV)		
		10	100	500
number of animals tested	(11)	(5)	(4)	(3)
Mount Frequency F (3,18) = 0.954, p = 0.4355	8 ± 5	13 ± 8	8 ± 3	8 ± 6
Intromission Frequency F (3,19) = 5.626, p = 0.0801	14 ± 4	18 ± 3	10 ± 1	10 ± 3
Mount Latency F (3,19) = 1.937, p = 0.1578	51 ± 64	189 ± 113	40 ± 5	6 ± 1
Intromission Latency F (3,19) = 1.564, p = 0.2310	89 ± 92	256 ± 164	45 ± 4	17 ± 4
Ejaculation Latency F (3,19) = 3.619, p = 0.0321	432 ± 205	762 ± 214 *	285 ± 29	203 ± 62
Post Ejaculatory Interval F (3,19) = 2.173, p = 0.1247	339 ± 53	371 ± 28	415 ± 29	388 ± 17
Hit Rate F (3,19) = 0.302, p = 0.8232	65 ± 17	60 ± 4	57 ± 3	59 ± 14
Intercopulatory Interval F (3,19) = 0.614, p = 0.6144	24 ± 8	29 ± 2	25 ± 3	21 ± 10
Copulatory Duration F (3,19) = 3.914, p = 0.0248	342 ± 144	506 ± 90	240 ± 31	186 ± 61

Note. Data shown are means ± standard errors. The number of animals tested in each group equaled the number of animals who displayed male copulatory behavior (n). Only one behavioral test was given 13 days after implant surgery. One-way ANOVA statistics are listed below the parameter names. Asterisks indicate $p < 0.05$ vs. the testosterone group, Fisher's PLSD Post-hoc Test.

Table 19. Effects of RU 58668 on Male Copulatory Behavior

	two, 10 mm testosterone capsules			
		RU 58668 (mg/kg/day sc)		
	Test	3	15	
number of animals tested	(16)	(8)	(10)	
Mount Frequency	0	4 ± 1 (9)	3 ± 1 (7)	4 ± 1 (7)
F (4,39) = 2.602	1	8 ± 1 (12)	11 ± 3 (4)	3 ± 1 (7)
p = 0.0507	2	5 ± 1 (16)	5 ± 1 (7)	7 ± 2 (10)
Intromission Frequency	0	9 ± 1 (9)	9 ± 1 (7)	8 ± 1 (7)
F (4,39) = 1.967	1	14 ± 2 (12)	12 ± 3 (4)	7 ± 1 (7)
p = 0.1187	2	10 ± 1 (16)	15 ± 3 (7)	10 ± 2 (10)
Mount Latency	0	47 ± 33 (9)	56 ± 42 (7)	89 ± 57 (7)
F (4,39) = 0.863	1	71 ± 40 (12)	24 ± 12 (5)	118 ± 65 (8)
p = 0.4947	2	69 ± 30 (16)	103 ± 66 (7)	189 ± 90 (10)
Intromission Latency	0	50 ± 33 (9)	58 ± 42 (7)	104 ± 58 (7)
F (4,39) = 0.878	1	81 ± 41 (12)	35 ± 14 (5)	133 ± 74 (7)
p = 0.4862	2	84 ± 35 (16)	108 ± 66 (7)	217 ± 111 (10)
Ejaculation Latency	0	217 ± 45 (9)	211 ± 57 (7)	263 ± 77 (7)
F (4,39) = 2.859	1	450 ± 92 (12)	852 ± 205 (5) *	220 ± 84 (7)
p = 0.0360	2	277 ± 51 (16)	346 ± 69 (7)	512 ± 153 (10) *
Post Ejaculatory Interval	0	336 ± 15 (9)	316 ± 10 (7)	321 ± 25 (7)
F (4,39) = 1.250	1	362 ± 33 (12)	347 ± 52 (4)	400 ± 62 (7)
p = 0.3061	2	342 ± 14 (16)	378 ± 17 (7)	359 ± 17 (10)
Hit Rate	0	72 ± 4 (9)	77 ± 5 (7)	72 ± 5 (7)
F (4,39) = 2.987	1	64 ± 3 (12)	54 ± 8 (5)	75 ± 6 (7)
p = 0.0304	2	69 ± 3 (16)	75 ± 3 (7)	62 ± 5 (10)
Intercopulatory Interval	0	18 ± 3 (9)	16 ± 2 (7)	18 ± 3 (7)
F (4,39) = 7.866	1	24 ± 3 (12)	83 ± 26 (5) *	12 ± 2 (7)
p = 0.0001	2	18 ± 1 (16)	17 ± 1 (7)	32 ± 6 (10) *
Copulatory Duration	0	168 ± 29 (9)	152 ± 34 (7)	160 ± 34 (7)
F (4,39) = 5.013	1	369 ± 91 (12)	816 ± 200 (5) *	87 ± 22 (7) *
p = 0.0023	2	194 ± 28 (16)	238 ± 33 (7)	295 ± 75 (10)

Note. Data shown are means ± standard errors. The numbers in parentheses next to the standard errors are the number of animals displaying the behavior (n). Test 0 was the pretest before gonadectomy, Test 1 was 7 days after implant surgery, and Test 2 was 13 days after implant surgery. Repeated Measures ANOVA statistics for the group x test interaction are listed below the parameter names. Asterisks indicate $p < 0.05$ vs. the testosterone group for each test, single degree of freedom contrast test on group means.

Table 20. Male Copulatory Behavior - Hydroxyflutamide Pilot Study

	two, 10 mm testosterone capsules			
	hydroxyflutamide (µg/day ICV)			
	2.5	25.4	250	750
number of animals tested	(11) (3)	(4)	(3)	(3)
Mount Frequency	8 ± 1	11 ± 3	8 ± 2	18 ± 8	8 ± 2
F (5,22) = 1.307, p = 0.2970					
Intromission Frequency	14 ± 1	17 ± 3	9 ± 1	21 ± 7	14 ± 3
F (5,22) = 1.909, p = 0.1336					
Mount Latency	51 ± 19	69 ± 33	35 ± 9	226 ± 125	120 ± 70
F (5,22) = 2.171, p = 0.0945					
Intromission Latency	89 ± 28	75 ± 36	52 ± 14	230 ± 130	120 ± 70
F (5,22) = 1.322, p = 0.2916					
Ejaculation Latency	432 ± 62	561 ± 152	301 ± 44	768 ± 440	415 ± 99
F (5,22) = 0.789, p = 0.5691					
Post Ejaculatory Interval	339 ± 16	356 ± 9	336 ± 25	438 ± 27	420 ± 37
F (5,22) = 3.505, p = 0.0176					
Hit Rate	65 ± 5	61 ± 5	56 ± 9	56 ± 3	63 ± 8
F (5,22) = 0.335, p = 0.8860					
Intercopulatory Interval	24 ± 2	27 ± 3	30 ± 8	22 ± 6	23 ± 4
F (5,22) = 0.444, p = 0.8129					
Copulatory Duration	342 ± 43	486 ± 133	249 ± 31	538 ± 314	296 ± 29
F (5,22) = 0.707, p = 0.6243					

Note. Data shown are means ± standard errors. The number of animals tested equaled the number of animals who displayed male copulatory behavior (n). Only one behavioral test was given 13 days after implant surgery. One-way ANOVA statistics are listed below the parameter names. ICV administration of hydroxyflutamide did not have any significant effect on male copulatory behavior.

Table 21. Effects of ICV Delivery of Hydroxyflutamide on Male Copulatory Behavior

		two, 10 mm testosterone capsules	
hydroxyflutamide			2 mg/day ICV
number of animals tested		(16)	(10)
	Test		
Mount Frequency			
t (15) = 0.546, p = 0.5932	1	8 ± 1 (12)	10 ± 3 (5)
t (22) = 0.475, p = 0.6394	2	5 ± 1 (16)	6 ± 1 (7)
Intromission Frequency			
t (15) = -1.447, p = 0.1684	1	14 ± 2 (12)	10 ± 1 (5)
t (21) = -0.274, p = 0.7866	2	10 ± 1 (16)	10 ± 2 (7)
Mount Latency			
t (15) = 1.573, p = 0.1365	1	71 ± 40 (12)	225 ± 120 (5)
t (21) = -0.981, p = 0.3376	2	69 ± 30 (16)	24 ± 10 (7)
Intromission Latency			
t (15) = -1.531, p = 0.1465	1	81 ± 41 (12)	237 ± 127 (5)
t (21) = -0.708, p = 0.4865	2	84 ± 35 (16)	45 ± 13 (7)
Ejaculation Latency			
t (15) = 0.011, p = 0.9916	1	450 ± 92 (12)	451 ± 169 (5)
t (21) = -0.461, p = 0.6493	2	277 ± 51 (16)	238 ± 53 (7)
Post Ejaculatory Interval			
t (15) = 1.240, p = 0.2341	1	362 ± 33 (12)	435 ± 47 (5)
t (21) = -0.132, p = 0.8964	2	342 ± 14 (16)	338 ± 17 (7)
Hit Rate			
t (15) = -1.512, p = 0.1514	1	64 ± 3 (12)	54 ± 8 (5)
t (21) = -0.446, p = 0.6605	2	69 ± 3 (16)	66 ± 6 (7)
Intercopulatory Interval			
t (15) = 0.025, p = 0.9807	1	24 ± 3 (12)	24 ± 6 (5)
t (21) = 0.364, p = 0.7196	2	18 ± 1 (16)	19 ± 3 (7)
Copulatory Duration			
t (15) = -1.046., p = 0.3122	1	369 ± 91 (12)	215 ± 49 (5)
t (21) = -0.023, p = 0.9816	2	194 ± 28 (16)	192 ± 45 (7)

Note. Data shown are means ± standard errors. The numbers in parentheses below the standard errors are the number of animals displaying the behavior (n). Test 1 was 7 days after implant surgery, and Test 2 was 13 days after implant surgery. One-way ANOVA statistics for each test are listed below the parameter names. Repeated Measures ANOVAs were not performed because a pretest was not done, and thus, baseline data was not available. Asterisks indicate $p < 0.05$ vs. the testosterone group, Fisher's PLSD Post-hoc Test. Asterisks indicate $p < 0.05$ vs. the testosterone group, Fisher's PLSD Post-hoc Test.

Table 22. Effects of Hydroxyflutamide with, or without, RU 58668 on Male Copulatory Behavior

		two, 10 mm testosterone capsules		
hydroxyflutamide			40 mg/Kg sc	40 mg/Kg sc
RU 58668				15 mg/kg sc
number of animals tested		(16)	(14)	(14)
	Test			
Mount Frequency	0	4 ± 1 (9)	3 ± 1 (10)	3 ± 1 (6)
F (4,30) = 3.045	1	8 ± 1 (12)	15 ± 1 (2) *	23 ± 8 (4)
p = 0.0321	2	5 ± 1 (16)	12 ± 2 (5)	12 ± 5 (4)
Intromission Frequency	0	9 ± 1 (9)	8 ± 1 (10)	7 ± 1 (6)
F (4,28) = 0.997	1	14 ± 2 (12)	7 ± 2 (2)	8 ± 1 (4)
p = 0.4257	2	10 ± 1 (16)	11 ± 2 (4)	7 ± 1 (3)
Mount Latency	0	47 ± 33 (9)	27 ± 17 (10)	7 ± 3 (6)
F (4,30) = 1.373	1	71 ± 40 (12)	132 ± 119 (3)	63 ± 23 (4)
p = 0.2667	2	69 ± 30 (16)	144 ± 116 (4)	148 ± 111 (4)
Intromission Latency	0	50 ± 33 (9)	33 ± 17 (10)	14 ± 3 (6)
F (4,28) = 2.470	1	81 ± 41 (12)	305 ± 283 (2)	391 ± 269 (4)
p = 0.0676	2	84 ± 35 (16)	156 ± 115 (4)	94 ± 43 (3)
Ejaculation Latency	0	217 ± 45 (9)	194 ± 33 (10)	113 ± 25 (6)
F (4,27) = 1.828	1	450 ± 92 (12)	762 (1)	794 ± 246 (4)
p = 0.1526	2	277 ± 51 (16)	441 ± 182 (4)	305 ± 110 (3)
Post Ejaculatory Interval	0	336 ± 15 (9)	334 ± 26 (10)	278 ± 18 (6)
F (4,26) = 3.854	1	362 ± 33 (12)	546 (1) *	421 ± 72 (3)
p = 0.0137	2	342 ± 14 (16)	405 ± 91 (4)	613 ± 169 (3) *
Hit Rate	0	72 ± 4 (9)	74 ± 4 (10)	69 ± 3 (6)
F (4,28) = 5.560	1	64 ± 3 (12)	30 ± 4 (2) *	30 ± 9 (4) *
p = 0.0020	2	69 ± 3 (16)	45 ± 4 (4) *	43 ± 11 (3) *
Intercopulatory Interval	0	18 ± 3 (9)	15 ± 2 (10)	20 ± 2 (6)
F (4,26) = 2.442	1	24 ± 3 (12)	22 (1)	54 ± 27 (4)
p = 0.0720	2	18 ± 1 (16)	32 ± 8 (3)	30 ± 19 (3)
Copulatory Duration	0	168 ± 29 (9)	99 ± 23 (10)	162 ± 23 (6)
F (4,27) = 0.357	1	369 ± 91 (12)	174 (1)	403 ± 218 (4)
p = 0.8370	2	194 ± 28 (16)	285 ± 71 (4)	211 ± 136 (3)

Note. Data shown are means ± standard errors. The numbers in parentheses below the standard errors are the number of animals displaying the behavior (n). Test 1 was 7 days after implant surgery, and Test 2 was 13 days after implant surgery. Repeated Measures ANOVA statistics for the group x test interaction are listed below the parameter names. Asterisks indicate $p < 0.05$ vs. the testosterone group for each test, single degree of freedom contrast test on group means.

Appendix B - Partner Preference Computer Program Manual

Program Requirements:

The program will run on any Apple Macintosh computer (including Power Macintoshes) running Macintosh Operating System 6.0.5 or later (including Mac OS 8.0) with at least 1 Mb of RAM. This includes Macintosh Plus and SE computers.

Starting:

Double-click the program's icon to start. The program immediately opens a window set up for two, three chambered place preference tests (see Figure 67). This version of the program only supports up to two, three chambered tests. The default test time for both chambers is ten minutes. The user can change the test time for all of the timers by pulling down the settings menu and selecting "Test Time" where one of the predefined times can be selected, or the user can input their own (*Note*: changing a program setting resets both counters.). To begin the test, use the mouse to click the "Start Test" button for each timer. The computer then begins counting down, in seconds, from the selected test time. The program defaults that the test subject is located in the middle chamber at the beginning of the test.

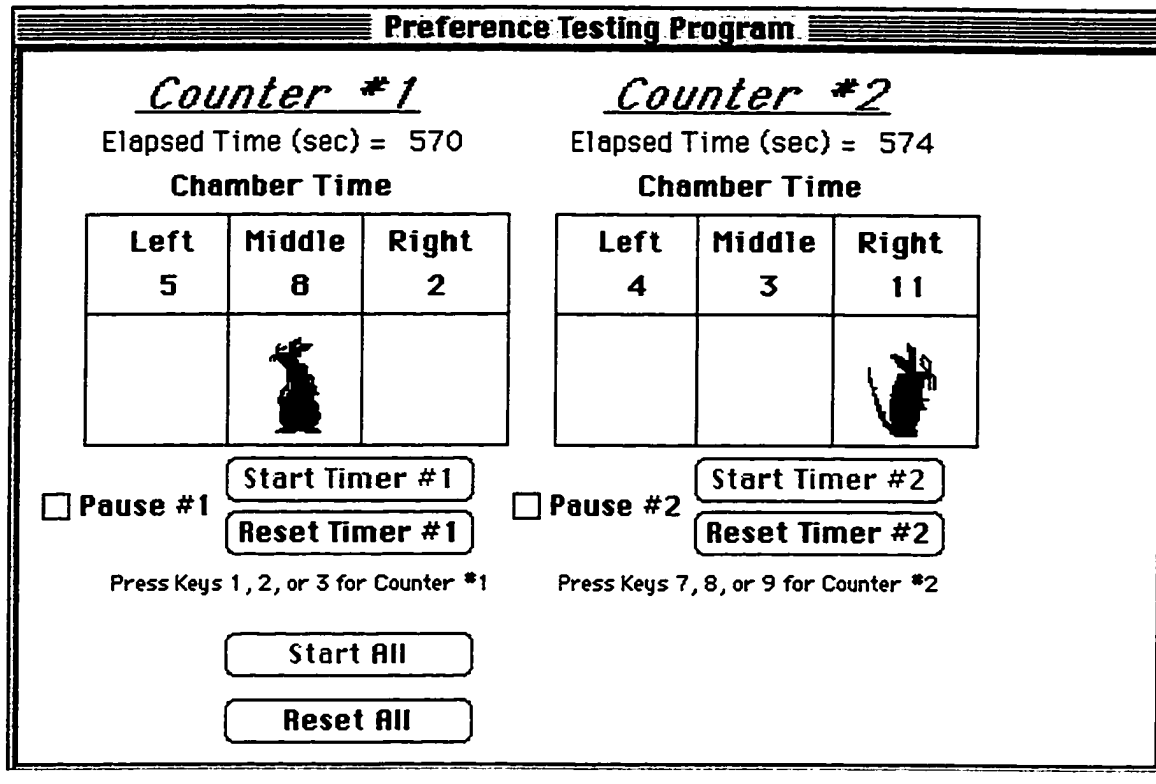


Figure 67. Partner Preference Program Main Window

Operation:

When an animal is observed to move into another chamber, the user then presses a key to notify the computer that the animal has crossed a chamber boundary. The program has been set up so that for timer one, the left, middle, and right chambers are denoted by the keys "1, 2, and 3" respectively. For timer two, the left, middle, and right chambers are denoted by the keys "7, 8, and 9" respectively. For example, at the start of the test the animal is in the middle chamber. Then lets say that after ten seconds the animal moves into the left chamber. When the animal crosses the left/middle chamber boundary, the user would hit the "1" key. The amount of time the animal just spent in the middle chamber is automatically calculated and displayed during the test. Then lets say the animal spends twenty seconds in the left chamber and then moves back to the middle chamber. The user

would then press the "2" key to indicate that the animal has moved back to the middle chamber. The computer would then update the display for the time spent in each chamber (which would now read 20 sec left, 10 sec middle, 0 sec right). The user would then proceed to track the animals movements during the duration of the test. If there is a problem during the test, the user can pause each timer individually by selecting the "Pause check box" with the mouse, correct the problem, and the deselect the "Pause check box" to continue with the test. When the individual timers reach zero time remaining the user is notified by a sound for each timer.

During the test, the user can view additional statistics by pulling down the "Movement Statistics" menu (see Figure 68). This window provides the number of visits to each chamber and the average amount of time spent in each chamber.

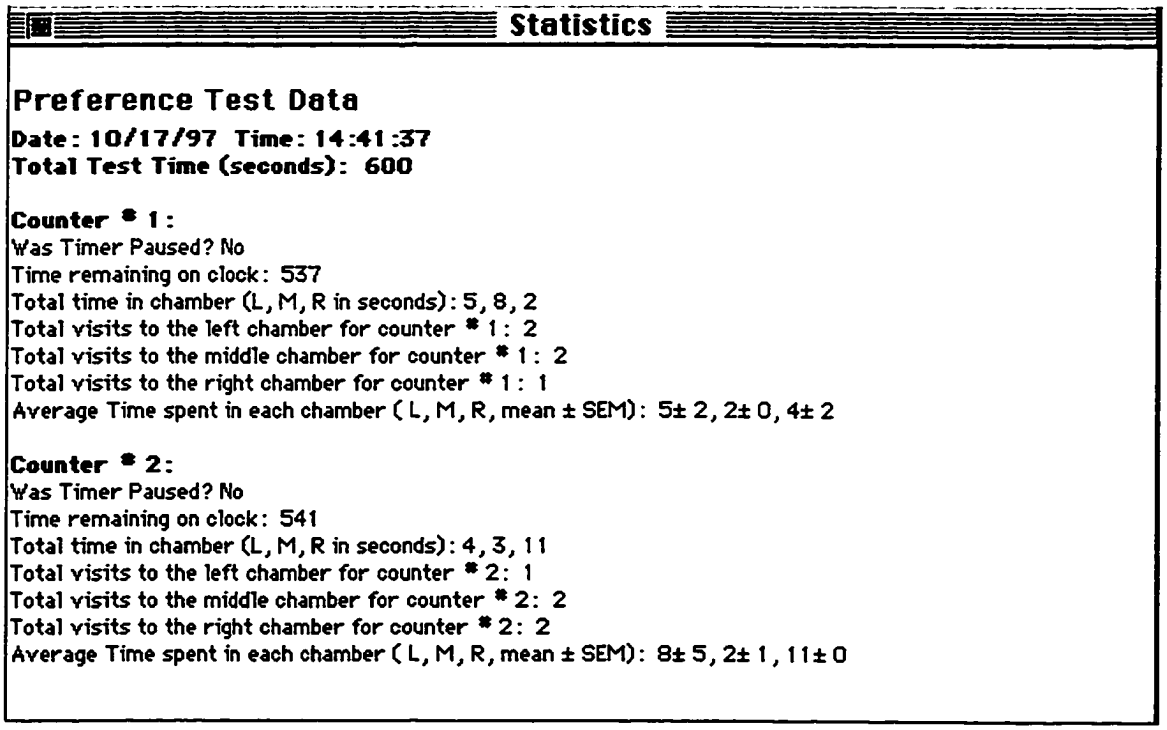


Figure 68. Movement Statistics Window

Output:

The user can save the data to a new text file, append the data to an old text file, copy the data to the clipboard, print the data, or just write the results down on paper (*Note*: Replacing a file using the "Save" option will erase the old file, while replacing a file using the "Append" option will add the results to a previously saved text file.). For either saving, printing, or copying the results, the user is prompted to input text information, such as the animal's number for each test apparatus.

The data files written to disk are ASCII text files with the results separated by tabs. These files can be opened with any program that can read text files such as word processing programs or spreadsheets (such as Microsoft Excel). The program provides the user with a choice of two file types. The default is the "Short Form", which mainly provides the total time the animal spends in each chamber and the animals ID (see Figure 69). The other type of output is the "Long Form", which consists of the test date and time, user provided text information, the total time spent in each chamber, and the following additional statistics, the total number of chamber visits to each chamber and the average time per visit spent in each chamber plus its standard error of the mean (see Figure 70).

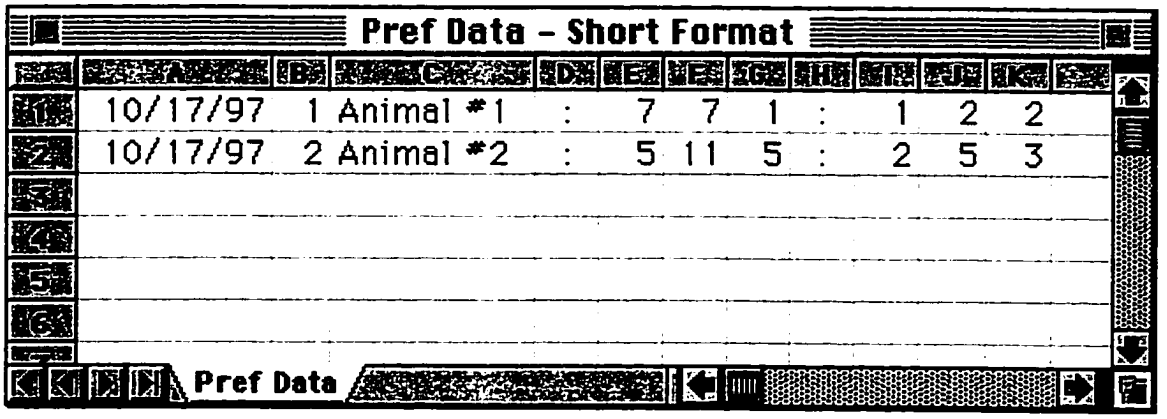


Figure 69. Preference Program Data Output - Short Form

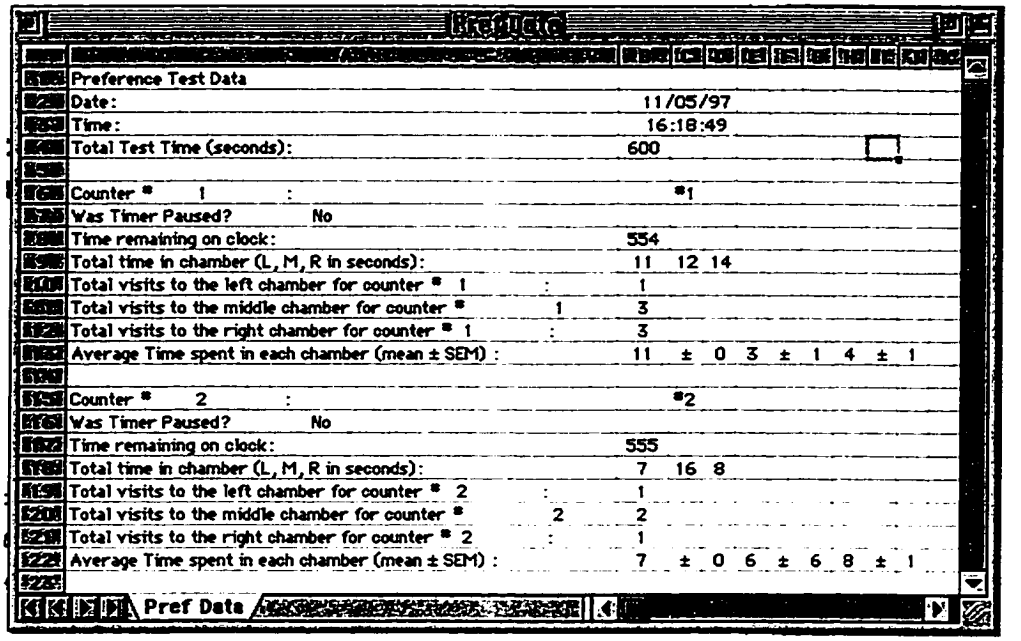


Figure 70. Preference Program Data Output - Long Form

Resetting:

To test more animals, the user just needs to select the "Reset button" for each timer, and the program will be ready to start over.

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IMAGE EVALUATION
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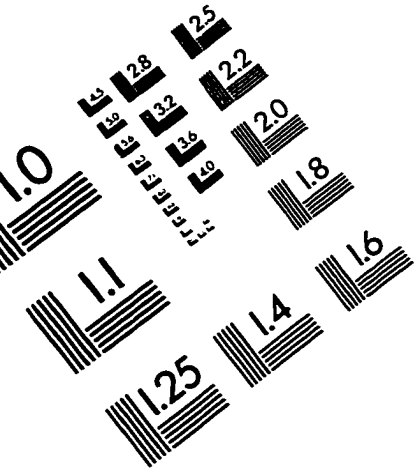

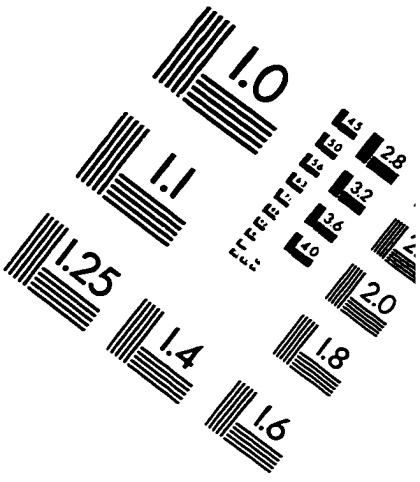

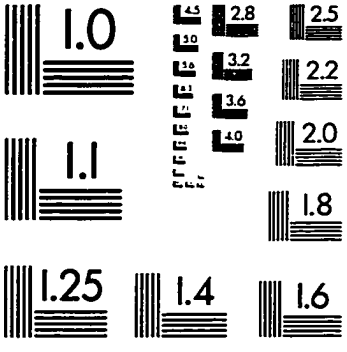

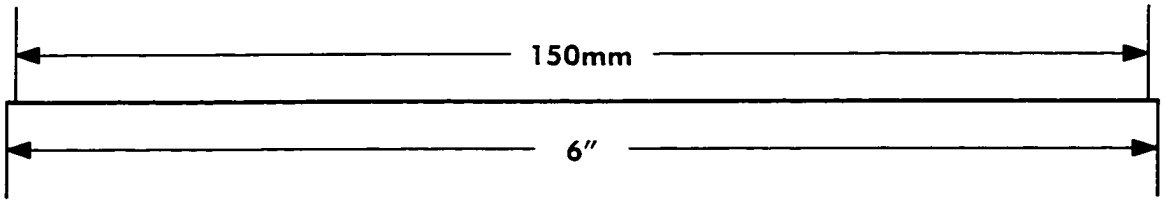

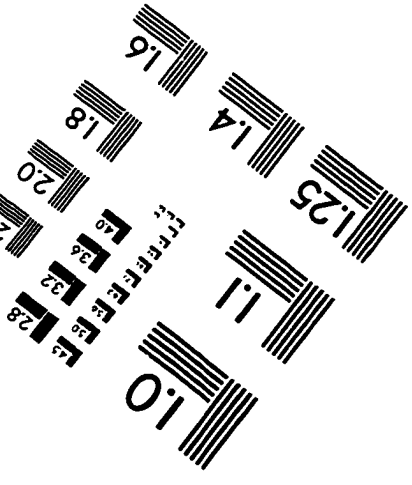

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