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PRENATAL INFLUENCES OF ANDROGEN UPON
THE DEVELOPMENT OF SEXUAL BEHAVIOR
IN THE RAT (RATTUS NORVEGICUS)

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PRENATAL INFLUENCES OF ANDROGEN UPON THE DEVELOPMENT OF SEXUAL BEHAVIOR IN THE RAT (RATTUS NORVEGICUS)

By

Brian Anthony Gladue

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ABSTRACT

PRENATAL INFLUENCES OF ANDROGEN UPON
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The neonatal organization of patterns of adult sexual behavior is thought to be under androgenic control. This androgen activity organizes masculine sexual behavior (masculinization) and blocks the organization of feminine sexual behavior potential (defeminization). Feminization, the organization of feminine behavior patterns, is thought to occur in the absence of gonadal hormone stimulation.

In the rat, the developmental period in which the nervous system and genitalia are organized by androgens was previously thought to occur postnatally. Prenatal influences of hormone action upon development had been implicated but have not been thoroughly explored. In this study, prenatal influences of androgen on the organization of masculine and feminine sexual behavior was examined in both male and female rats.

In Experiment 1 pregnant females were administered either 1 mg/day or 5 mg/day of the anti-androgen flutamide (4'-nitro-3'-trifluoromethyl-isobutyryanilide; SCH 13521; SCH) during days 10 to 22 of gestation (in a gestation period of 22 days). This time period overlaps considerable prenatal androgen secretory activity in fetal male rats. Blocking the organizing action of the androgen testosterone in the fetus via flutamide should result in offspring with less masculine sexual behavior. Males born to flutamide-treated mothers showed significantly decreased levels of copulatory behavior when castrated as adults and given daily testosterone propionate (100 ug) replacement therapy. In addition, female

offspring prenatally exposed to flutamide also showed diminished masculine sexual behavior as adults, suggesting that prenatal androgen secreted by males in utero influences masculine behavioral organization of neighboring females. Prenatal flutamide treatment also abolished sex differences in ano-genital distance at birth, indicating a potent anti-androgenic action of flutamide on the development of such secondary sex characteristics as penile development.

In Experiment 2 the prenatal influence of androgen upon the development of feminine sexual behavior was also investigated. Flutamide administered to pregnant rats from days 10 to 22 of gestation in dosages of either 1 mg/day or 5 mg/day resulted in offspring which displayed significantly greater levels of female receptivity (lordosis) in adulthood when tested in response to adult ovarian hormone replacement therapy. Males exposed to either dosage of flutamide exhibited significantly higher lordosis quotients than controls in tests with estradiol benzoate (EB) treatment only. The addition of progesterone (P) had no overall facilitatory effect on males with regard to estrogen-induced lordosis. Females exposed to flutamide prenatally showed significantly greater lordosis quotients than controls when tested over a variety of EB doses in adulthood. Addition of P to EB-treated females significantly facilitated lordosis display in control and flutamide-treated females.

The increase in feminine sexual behavior in both male and female rats resulting from prenatal anti-androgen treatment strongly indicates that androgen prenatally inhibits the development of feminine sexual behavior. This androgenic inhibition of receptivity (defeminization) appears to be related to changes in the animal's adult sensitivities to estrogen as a consequence of prenatal treatment. Further, some prenatal

androgen organization of masculine sexual behavior (masculinization) occurs in both sexes. Together these findings indicate significant prenatal development of sexual behavior patterns in both sexes and that feminine sexual behavior may be organized prior to masculinization.

"What is man that his welfare be considered?
An ape who chatters of kinship with the
archangels,
While he very filthily digs for groundnuts.
And yet I perceive that this same man is a
maimed god.
He is condemned under penalty
to measure Eternity with an hourglass,
and Infinity with a yardstick,
And what is more,
He very nearly does it."

- James Branch Cabell

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INTRODUCTION

Sexual differentiation of reproductive behavior and gonadotropin secretory activity in laboratory rats occurs as a result of gonadal hormone action perinatally. Historically, the investigation of this process has largely concentrated upon developmental events beginning after birth. Early work by endocrinologists such as Moore and Price (1932) advanced the view that estrous cycle changes in ovarian hormones are related to changes in the pituitary-gonadal axis. In 1936 Pfeiffer was the first to demonstrate the effects of neonatal gonadectomy and transplantation of gonads upon gonadotropin cyclicity in male and female rats. Of particular importance was the finding that castrated males showed normal ovarian cyclicity when given ovaries as adults; intact males did not. Further, females transplanted with testes at birth did not cycle as adults. The conclusion drawn was that an undifferentiated hypophyseal system is capable of secreting gonadotropins in a cyclical fashion. Intervention by testicular secretions, later determined to be androgen, primarily testosterone, shifted this inherently cyclical "female" system toward a noncyclic "male" secretory pattern.

The extension of these early findings toward implicating neural areas controlling the pituitary was synthesized by Harris in 1955. He expressed the view that sexual differentiation of the nervous system regulating gonadotropin secretion occurs in neonatal life with

the action of testosterone in the male organizing masculine patterns of gonadotropin secretion.

The extension of this concept of neonatal differentiation of adult gonadotropin secretory patterns to include adult sexual behavior was begun by Phoenix and his colleagues (Phoenix, Goy, Gerall and Young,1959) and later extended by Barraclough and Gorski (1962). Harris summarized the area in 1964 and suggested that masculinizing effects of testosterone or the feminizing consequences of no androgen may theoretically extend into prenatal life as well as the immediate postnatal development period (Harris, 1964; Harris and Levine, 1965). That is, while most studies of sexual differentiation between 1959 and 1964 had concentrated upon events occuring during early postnatal life, prenatal influences could not theoretically be ruled out. Harris' early suggestions of influences occuring prenatally during sexual differentiation is schematically depicted in Figure 1.

Since that time when Pfeiffer initiated a new approach to the dichotomy of gonadotropin hormone secretion between males and females, and the proposal of behavioral sex differentiation by Phoenix et al (1959), investigation has focused on hormonal influences occuring during early postnatal development. Following early exploratory studies by Beach (1942), Beach and Rasquin (1942) and Koster (1943), subsequent studies have revealed two major behavioral systems that are influenced by hormone during a critical developmental period: a system which promotes the display of feminine sexual behavior in response to adult ovarian hormones (feminization), and a system which promotes the display of masculine copulatory patterns in response to adult androgen treatment (masculinization). This latter system,

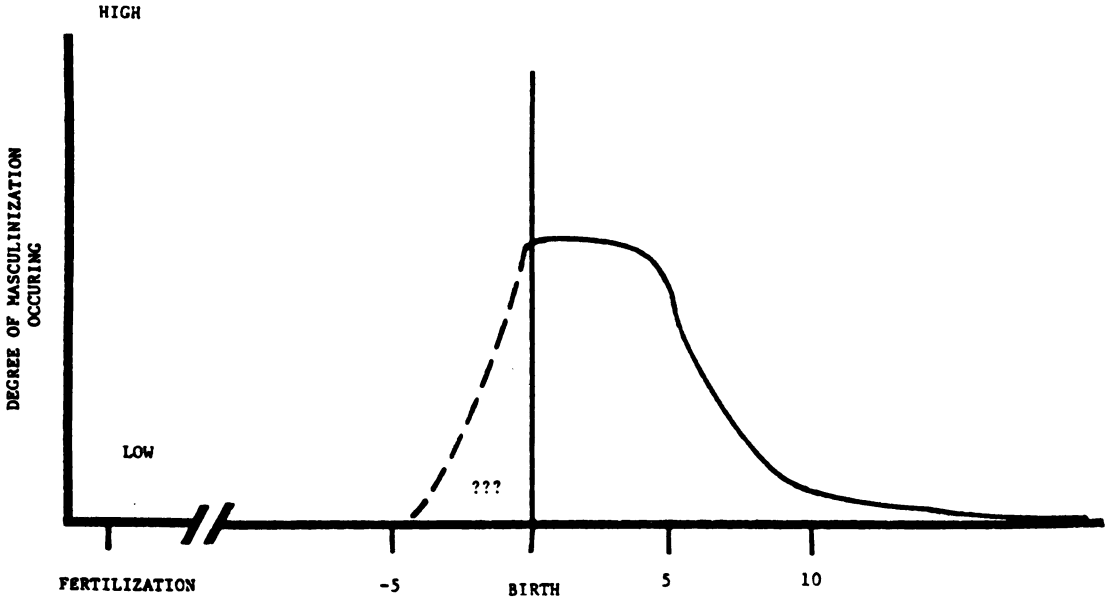

Figure 1. Diagram depicting critical period for sexual differentiation in the rat. Dashed line indicates theoretical (unknown) phase of developmental influences (after Harris, 1964).

masculinization, appears to result from androgenic exposure during early development, a period which until now was considered in the rat to be predominantly postnatal. The feminizing system appeared to develop postnatally and was susceptible to suppression by postnatal androgen treatment. Although prenatal influences of testosterone on masculinizing and feminizing systems of the guinea pig, a species with a long gestation phase and most of its development occuring prenatally, had been reported (Phoenix et al, 1959), investigations into prenatal aspects of sexual differentiation for other species lagged, perhaps since the period for defeminization in the rat was reported to be around 25 days post-fertilization, that is, three days postpartum (Goy, Phoenix and Young, 1962).

However, while postnatal treatments demonstrated the potent masculinizing actions of either endogenous androgens in males or exogenous androgen in females (Phoenix, Goy and Young, 1967; Gerall, 1967; Whalen and Edwards, 1967; Whalen, 1968) such treatments have not been capable of totally suppressing female sexual behavior in either sex. Males castrated at birth showed less sexual receptivity than normal females, suggesting some prenatal influences partially and possibly initially defeminizing those males (Gerall, Hendricks, Johnson and Bounds, 1967; Hendricks, 1969). Additionally, normal female rats show considerable mounting behavior as adults when given testosterone in adulthood (Ball, 1940; Beach, 1942; Beach and Rasquin, 1942; for review, see Beach, 1971), suggesting either a genetic predisposition to some masculine behavior or some prenatal non-genetic (hormonal) masculinizing influence upon their development. Since androgen action around the time of birth appears to permanently

organize sexual behavior patterns and neuroendocrine secretory patterns of the adult (Barraclough, 1967; Harris and Levine, 1965; Gorski, 1971) it was suspected that some prenatal androgenic activity on behavioral and neuroendocrine organization might account for the failure to achieve a total male or female pattern with postnatal treatments alone.

In the rat, behavioral sex differentiation is preceded by morphological sex differentiation. Starting at about day 10 of gestation (in a 22 day gestation period) the fetal gonad begins to differentiate into either a testis or ovary. Testicular development is thought to be a consequence of the gonadal organizing action of H-Y antigen (histocompatibility antigen Y), a chemical instruction located on the Y-chromosome (Ohno, 1976; 1977). In the absence of H-Y antigen the presumptive gonad differentiates into an ovary and the primordial genital duct tissues develop as the Mullerian reproductive system. In the presence of H-Y antigen, the gonadal primordium yields a testis which begins secreting androgens, primarily testosterone, which further develop the mesonephric tubule system toward the Wollfian (male) reproductive tract. A second substance, the unidentified peptide known as Mullerian Inhibiting Factor (MIF) is secreted which dismantles the primordial female reproductive tract.

Thus, the general mammalian pattern of development occurs in two stages. Initially, the gonad organizes into a testis or ovary, probably resulting from the presence or absence of H-Y antigen. The testis then begins secreting further organizing (androgenic) and disorganizing (MIF) substances. At approximately day 18 of gestation testosterone levels, which had begun to appear at around day 14 of gestation, reach

peak levels (Picon, 1976; Picon and Ktorza, 1976). These fetal testicular secretions gradually decline toward the onset of parturition (Sanyal and Villee, 1977). In contrast, these authors report little or no fetal ovarian activity, although recently, fetal estrogen production has been found in the rabbit (Millewich, George and Wilson, 1977). Key events in the sexual development of the rat occuring during and shortly after gestation are indicated schematically in Figure 2 , illustrating the considerable opportunity for sexual differentiation to occur prenatally given the degree and duration of androgen presence before birth.

Since the testis is active prenatally and since previous investigators have suspected androgens occuring prenatally exert organizing influences on the behavior and genital morphology of the female and male rat, the possible influences these androgens might exert upon masculine and feminine sexual behavior differentiation was investigated.

Normal female rats are capable of showing mounting and intromission patterns characteristic of normal males. Given testosterone perinatally female rats achieved higher levels of these male-like responses including the display of the ejaculatory pattern than did normal females (Whalen and Edwards, 1967; Whalen and Robertson, 1968; Ward, 1969). And in males, treatment perinatally with the steroidal antiandrogen cyproterone acetate (CA) reduced the frequency of intromission and ejaculation (Nadler, 1969; Ward and Renz, 1972). These changes in adult masculine behavior, a result of perinatal variations in androgens, lends support to the concept that male sexual behavior undergoes a period of androgen dependent organization (Phoenix et al, 1959). However, it has also been argued that the central neural system

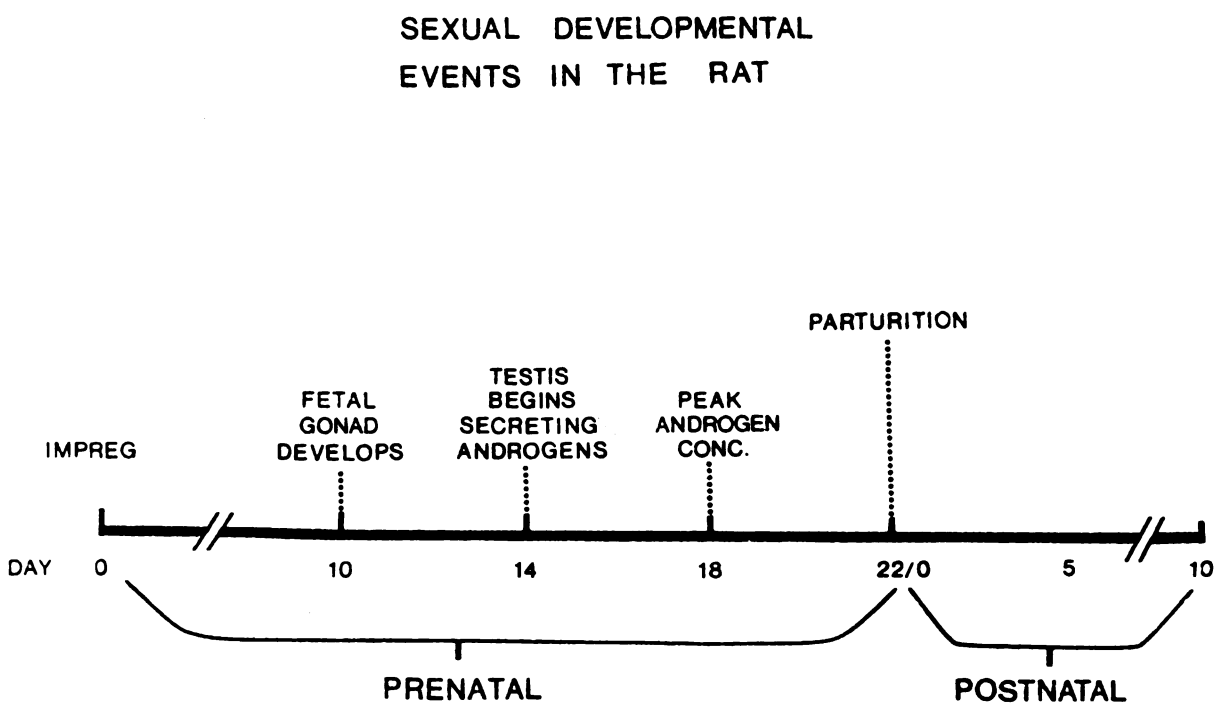

Figure 2. Schematic diagram depicting events in the sexual development of the rat.

mediating masculine sexual behavior may be organized without developmental androgenic influences and that the actions of androgen perinatally may best be seen as leading to development of peripheral genital tissues such as the penis, which then increases the probability of intromission responses (Whalen and Edwards, 1967; Nadler, 1969; Whalen, Edwards, Luttge and Robertson, 1969; Beach, 1971).

A central issue in trying to resolve these different interpretations of mounting behavior is whether or not the occurence of mounting and intromission in the normal female rat can be taken as evidence for development of masculine behavior in the absence of androgenic organizing influences. If the female rat is normally exposed to androgens during prenatal development then it is possible that her potential for masculine sexual behavior results from exposure to such androgens. On the other hand, if she is not normally exposed to androgens perinatally, then such potential may reflect normal genetic organization of masculine behavior in the absence of androgenic influence.

The consideration that partial masculinization in the female might occur prenatally and in the presence of androgens in the rat was suggested by Clemens (1974). By examining the intra-uterine location of females delivered by cesarian section, and testing those females for masculine sexual behavior in adulthood, a correlation of female mounting behavior and <u>in utero</u> proximity to a sibling male was obtained. A female situated between two males in the womb showed a greater probability of mounting as an adult. Females further away from males <u>in utero</u> were less likely to engage in masculine copulatory behavior. The notion that fetal androgen could influence neighboring females <u>in utero</u> was both intriguing and encouraging to investigators of prenatal

differentiation seeking an explanation for the apparent variable bisexual potential of the rat.

Defeminization, the process in which the potential for displaying feminine sexual behavior is restricted or otherwise permanently disrupted, is also considered to be a process under the influence of androgen action. The development of the potential for female sexual behavior is thought to occur in the absence of androgen stimulation and is thus inhibited by the presence of gonadal steroids during early development (Barraclough, 1967: Beach, 1971; 1975; Ward, 1974; Whalen, 1974). Additionally, feminine sexual behavior typical of females is rarely seen in normal male rats, presumably due to the perinatal action of androgen in removing the potential for receptivity in males. Male rats castrated shortly after birth showed an enhanced sensitivity to estrogen and progesterone as adults and will show high levels of receptivity (Gerall and Kenney, 1970; Pfaff and Zigmond, 1971; Hendricks, 1972; Sodersten, 1975; 1976). If androgen is responsible for the loss of feminine sexual behavior potential in males, and if such androgen exists prenatally in the male, it was hypothesized that androgen-induced defeminization may occur before birth as well as after birth in this species. Further, since fetal females may be exposed to androgen secreted from sibling males in utero, females may be partially defeminized prenatally as well. Males exposed in utero to cyproterone acetate showed higher quality feminine sexual behavior (lordosis) in adulthood than controls (Ward, 1972) as well as higher levels of overall sexual receptivity (Nadler, 1969; Ward, 1974).

The present study, then, examined the extent to which endogenous prenatal androgenic substnaces, particularly testosterone, may influence

the genital morphology and later masculine and feminine sexual behavior of both male and female rats. In order to interfere with endogenous androgens prenatally, a potent and highly specific anti-androgen was required. Earlier prenatal studies utilized the steroid cyproterone acetate (CA) to block possible androgen action (see review by Neumann and Steinbeck, 1974). However, treatment with CA requires delivery by cesarian section and subsequent cross-fostering of pups since CA has significant progestagenic activity blocking parturition and lactation (Neumann and Steinbeck, 1974; Ward and Renz, 1972). Further studies have also shown CA to be highly estrogenic and anti-estrogenic as measured by interference with hormonally mediated behaviors (Luttge, Hall, Wallis and Campbell, 1975; Luttge, 1975). The present study used a newer anti-androgen, the nonsteroidal compound flutamide (4'-nitro-3'-trifluoromethylisobutyrylanilide; SCH 13521; SCH) to inhibit prenatal androgen action. In contrast to CA, flutamide reportedly has little or no progestagenic or estroegnic side effects which might interfere with gestation and parturition (Neri, Florance, Koziol and Van Cleave, 1972; Neri and Peets, 1975; Neri, 1977; preliminary observations, Gladue and Clemens).

However, in order to fully interpret any behavioral organizing consequences this novel anti-androgen, flutamide, might exert via prenatal administration, the anti-hormonal capabilities of flutamide needed to be more fully characterized. Thus, experiments examining flutamide's capacity to have alternate hormonal or anti-hormonal actions other than as a specific anti-androgen were conducted in addition to experiments using this compound in prenatal investigations. Since these anti-hormonal characterization studies involved treating

adults and assessing influences on their sexual behavior and genital morphology, their results and discussion are handled seperately in the Appendix.

GENERAL METHODS

Animals

The Long-Evans rats (Rattus norvegicus) used in this study were maintained in standard laboratory conditions with a reverse dark-light cycle (10 h dark: 14 h light, lights off at 1100 h). Females used as mothers were obtained from a commercial breeder (Charles River Laboratories) at 90 days of age and were housed singly in plastic cages (15 X 30 X 34 cm).

Prenatal Treatments and Postnatal Procedures

Females impregnated in the laboratory (date of impregnation equals day 0 of gestation) were randomly assigned to one of three treatment conditions: a) flutamide (SCH) 1.0 mg/0.1 ml/day; b) SCH 5.0 mg/0.1 ml/day; c) propylene glycol (PROP) control vehicle, 0.1 ml/day. All pregnant females were administered the appropriate treatment injections daily (i.m.) from day 10 to day 22 of gestation when parturition normally occurs in this strain. Treatments were given to overlap and coincide with the suspected time of prenatal androgen secretion by fetal testes (Figure 2). Treatment did not interfere with either parturition or the ability to lactate or engage in normal parental behaviors. In addition, a control group of uninjected control mothers was employed in Experiment 1 to ascertain any potential effects of the propylene glycol vehicle on behavioral or morphological sex

differentiation. This control group was handled but not treated in any way with either injections or later neonatal surgical intervention.

All pups were sexed at birth (Day 1 of life), weighed and their ano-genital distance recorded. Ano-genital distance was measured with Vernier calipers under a dissecting microscope and is defined as the distance from the center of the anus to the posterior base of the genital tubercle.

Since flutamide abolished sex differences in genital morphology (preliminary observations), sexing was achieved by laparotomy to locate the testis or ovary (see surgical procedures).

Animals were weaned at 21 days of age and housed by sex and prenatal treatment condition in metal cages (18 X 36 X 50 cm) with 4 - 6 animals per cage. Males and females were gonadectomized in adulthood (70-90 days of age) and tested for the display of masculine sexual behavior at approximately 100 days of age. Testing for the display of feminine sexual behavior in males and females began at approximately 170 days of age.

Surgical Procedures

Neonatal laparotomy was employed to determine sex in those animals born to mothers given the anti-androgen flutamide. With the animal under cryo-anaesthesia , a ventral abdominal incision was made midway between the umbilical stump and genital tubercle. All operations were performed within twelve hours after birth. Animals exposed to the anti-androgen were examined for the presence of testis or ovary, while control animals (PROP) were sham-operated by locating the gonad only. Wounds were sealed with silk suture and flexible collodion and animals

were warmed on a heating pad before being returned to their respective mothers.

Adult gonadectomies were performed under ether anaesthesia when animals were 70-90 days of age. Males were castrated using a transscrotal approach, while females were ovariectomized with a bilateral flank approach. All wounds were sealed with silk and dissolvable sutures, followed by sterile wound clips on the exterior incision.

Behavioral Testing and Adult Hormone Treatments

Masculine Sexual Behavior

Six weeks after gonadectomy animals were given 3 weekly tests for the display of masculine sexual behavior. Tests were conducted by placing the experimental animal in a plexiglas testing arena (45 X 50 X 58 cm). Following a three minute adaptation period, a sexually receptive stimulus female was introduced into the test arena and the experimental animal was observed for the display of masculine sexual behavior until one of the following criteria was met : (a) elapse of twenty minutes from the introduction of the stimulus female without intromission (b) elapse of twenty minutes from intromission without ejaculation and (c) ejaculation followed by an intromission.

The stimulus female was made sexually receptive by injections of 10 ug estradiol benzoate (EB) given 72, 48 and 24 hrs before the test and 500 ug progesterone (P) 4 hrs before the test period.

In addition to mount and intromission frequencies a measure of copulatory behavior rate was computed by dividing time spent copulating (in minutes) into the sum of the mounts and mounts with intromission (MIPM: mounts and intromissions per minute). For animals not

ejaculating this was the time from the first mount (M) or intromission (I) to the last. For animals that achieved ejaculation time spent copulating was the latency from the first mount to ejaculation.

Following three weekly tests for baseline levels of sexual behavior animals were given hormone replacement therapy of 100 ug testosterone propionate (TP) daily (i.m.) and tested once per week for six weeks.

During each behavioral test, the occurence of mount, intromission and ejaculation pattern as well as the latencies to each of these events was recorded on an Esterline-Angus event recorder.

Feminine Sexual Behavior

Tests for feminine sexual behavior began 4 weeks after the end of tests for masculine copulatory behavior. Tests were conducted by placing the experimental animal in the plexiglas testing arena already occupied by a sexually vigorous stud male. Following treatment with ovarian hormones, adult animals were tested once weekly for the display of lordosis in response to mounting by the male. Lordosis is characterized by a deep ventral arching of the back with dorsal elevation of the head and lateral tail deflection. This behavioral response normally occurs in highly receptive females and is a potent measure of sexual receptivity. Lordosis behavior was quantified for each animal using the lordosis quotient: LQ= frequency of lordosis in response to 10 mounts/ 10 mounts X 100. Only those mounts with flank grasping and pelvic thrusting were used in calculating the lordosis quotient. Hormone treatments in adulthood used to elicit lordosis behavior are described below.

Hormone Treatments and Testing Regime

Males and females were tested for the display of lordosis in response to varying doses of estrogen with and without the addition of progesterone. In this way, estrogen sensitivity can be assessed as a result of prenatal treatments designed to inhibit defeminization. Further, by measuring lordosis with and without progesterone, prenatal treatment with anti-androgen can be measured with regard to influences on progesterone sensitivity.

The hormone regime consisted of single injections (i.m.) of estradiol benzoate (EB) 72, 48 and 24 hrs before testing. In tests conducted with progesterone, 500 ug of this steroid were administered 4 hrs before the start of behavioral testing. All behavior tests were given on the fourth day of hormone treatment (in the case of those tests without progesterone, no injection was given on the fourth day). All hormones were administered in 0.1 ml sesame oil.

Females

All females in the following groups: PROP (propylene glycol vehicle, n= 10), SCH 1 (flutamide-treated mothers received 1 mg/day, n= 7), SCH 5 (flutamide-treated mothers received 5 mg/day, n= 12) were tested with progesterone at EB dosages of 0.1 ug, 0.175 ug, 0.25 ug, 0.5 ug, 1.0 ug and 2.0 ug. All females were tested at the same dose level of EB each week. Tests for lordosis in which progesterone was not administered involved testing at dosage levels of 0.5 ug, 1.0 ug, 2.0 ug and 10.0 ug EB.

Males

The procedure regarding hormone treatment and testing of males differed from that of females in that only the 2.0 ug and 10.0 ug doses of EB were used. Further, males were tested for the display of lordosis three times at each dose (one test per week). This entire procedure was repeated with the addition of 500 ug progesterone given 4 hrs prior to the onset of behavioral testing as in the case for females.

Testing for sexual receptivity with progesterone occured first. Two weeks later tests without progesterone were begun.

Solutions

Steroid solutions were made by dissolving crystalline testosterone propionate, estradiol benzoate and progesterone (Schering Corporation, Bloomfield, New Jersey, USA) in sesame oil. Flutamide, a gift of Dr. R. O. Neri, also of Schering Corporation, was initially suspended in propylene glycol (1,2-propanediol; Sigma Chemical Corporation, St. Louis Missouri) and dissolved by mixing in a 55° C water bath. This latter tec hniq ue is capable of dissolving flutamide in concentrations up to 50 mg/ml.

Statistical Analyses

Behavioral data and morphological measures were analyzed with multiple and one-way analyses of variance. Whenever the F statistic indicated significance, group comparisons were made using a Student-Newman-Keuls procedure (Winer, 1971; Nie, Hull, Jenkins, Steinbrenner and Bert, 1975). Where appropriate, paired comparisons were analyzed with Student's t-test according to Nie, et al (1975).

EXPERIMENT 1. Prenatal Androgenic Influences on Masculine Sexual Behavior and Genital Morphology.

INTRODUCTION

If androgen is capable of organizing masculine sexual behavior in the rat, and if such androgen exists prenatally in the male via fetal testicular production, then prenatally occuring endogenous androgen may be the initial organizing influence toward overall masculinization of the organism. To examine this possibility, flutamide was administered to pregnant mothers and offspring of these mothers were tested for the display of masculine sexual behavior in adulthood. If the anti-androgen flutamide interfered with prenatally masculinizing androgen action, a decrease in adult mounting potential would be expected as well as diminished genital morphology and later intromission behavior.

Females were also tested to assess whatever in utero consequences endogenous androgen from sibling males might have upon the organization of their behavior potential.

METHOD

Treatment of pregnant females and the later testing of male and female offspring of these females is described earlier in the General Methods section.

RESULTS

Behavior

Masculine copulatory behavior in both males and females was affected by prenatal exposure to the anti-androgen flutamide. Intromission frequency was lower for flutamide treated males than for vehicle treated and uninjected controls ($F=14.401$, 3/39, $P < 0.001$) (Table 1). Males treated with the low dose of flutamide (SCH 1) achieved significantly more mounts than did those animals exposed to the high dose of flutamide (SCH 5) or either control group (SNK, $P< 0.05$). Examination of the rate of mounts and intromissions (MIPM) indicated that males treated with SCH 5 were significantly slower than either control group or the males prenatally exposed to SCH 1 (SNK, $P< 0.05$). Males treated with flutamide did not show any ejaculatory behavior.

Females exposed *in utero* to the high dose of flutamide (SCH 5) displayed significantly fewer mounts than did either control group ($F=3.015$, 3/40, $P < 0.05$; Table 2). Mounting rate was also decreased in females exposed to SCH 5 prenatally when compared to animals exposed to the lower dose of flutamide (SCH 1) or controls ($F=3.20$, 3/40, $P < 0.05$; Table 2). Intromission frequency was low in all female groups and consequently experimental females did not differ from controls ($F=1.985$, 3/40, $P< 0.1$).

Morphology

Prenatal exposure to flutamide resulted in profound morphological changes in the external genitalia at birth. Males born to mothers treated with either dose of flutamide revealed significantly shorter

Table 1. Mount Frequency (MF), Intromission Frequency (IF) and Mounting Intromission Rate (MIPM) in Males Prenatally Exposed to Flutamide (SCH 1.0 mg or SCH 5.0 mg), Propylene Glycol or Uninjected Controls.

Group	N	MF	IF	MIPM
SCH 1.0	10	37.91 ± 6.09*	0.36 ± 0.35*	1.69 ± 0.24
SCH 5.0	10	10.78 ± 5.29	0.55 ± 0.54*	0.45 ± 0.21*
Propylene Glycol	13	8.50 ± 2.71	8.60 ± 1.67	1.83 ± 0.34
Uninjected Controls	10	10.40 ± 2.07	11.30 ± 0.93	2.10 ± 0.37

All values represented as Mean ± S.E.M.

* $P < 0.05$, Student-Newman Keuls test, compared to both propylene glycol and uninjected controls.

Table 2. Mount Frequency (MF), Intromission Frequency (IF) and Mounting Intromission Rate (MIPM) in Female Rats Prenatally Exposed to Flutamide (SCH 1.0 and SCH 5.0), Propylene Glycol or Uninjected Controls.

Group	N	MF	IF	MIPM
SCH 1.0	7	18.86 ± 4.80	0.71 ± 0.36	1.00 ± 0.25
SCH 5.0	13	7.00 ± 2.23*	0.56 ± 0.38	0.38 ± 0.12*
Propylene Glycol	14	13.50 ± 2.82	0.54 ± 0.23	0.78 ± 0.16
Uninjected Controls	10	12.60 ± 2.60	0.20 ± 0.13	0.63 ± 0.13

All values represented as Mean ± S.E.M.

* $P < 0.05$, Student-Newman-Keuls test, compared to Propylene Glycol and Uninjected Controls.

ano-genital distances (AGD) when compared to either propylene glycol or uninjected controls ($F=330.9$, 4/58, $P < 0.001$; SNK, $P < 0.05$; Table 3). The AGD scores of SCH 5 males were within the range observed for normal female rats. No overall AGD differences as a result of prenatal treatment with flutamide were seen in females ($F=0.497$, 3/50, $P = 0.11$).

Body weight was unaffected by prenatal anti-androgen treatment in female offspring ($F=1.416$, 3/50, $P=0.23$) but there was a marginal but not statistically significant reduction in body weight of flutamide treated males when compared to PROP controls, while uninjected controls did not differ from flutamide-treated males or PROP males ($F=2.70$, 3/58, $P=0.06$; SNK, $P > 0.05$). The AGD in SCH 1 males appeared greater than that of comparably treated females. However, comparison of the different groups after adjustments for body weight had been made (AG/BW: ano-genital distance divided by body weight) revealed that males and females treated prenatally with either dose of flutamide were not significantly different in this measure of morphology (Table 3). Indeed, males treated with flutamide prenatally exhibited not only a penis in adulthood (albeit smaller than normal) but a patent blind vagina as well.

Table 3. Ano-Genital Distance, Body Weight and the Ratio of Ano-Genital Distance to Body Weight at Birth (AG/BW) in Males and Females Prenatally Exposed to Flutamide (SCH 1.0 or SCH 5.0), Propylene Glycol or Uninjected Controls.

GROUP	Ano-Genital Distance		Body Weight		AG/BW	
	Males	Females	Males	Females	Males	Females
SCH 1.0	1.77 ± 0.05 (12)*	1.57 ± 0.07(10)	5.93 ± 0.16	5.57 ± 0.09	0.298 ± 0.007	0.283 ± 0.013
SCH 5.0	1.66 ± 0.04 (20)*	1.66 ± 0.05(18)	5.96 ± 0.07	5.54 ± 0.07	0.278 ± 0.008	0.299 ± 0.008
Propylene Glycol	3.33 ± 0.05 (19)	1.64 ± 0.04(14)	6.27 ± 0.12	5.77 ± 0.18	0.535 ± 0.016*	0.287 ± 0.012
Uninjected Controls	3.34 ± 0.07 (11)	1.55 ± 0.05(12)	5.83 ± 0.11	5.43 ± 0.10	0.575 ± 0.017*	0.268 ± 0.010

All values represented as Mean ± S.E.M.
Numbers in parentheses indicate number of observations (animals) constituting the sample.
* $P < 0.05$, Student-Newman-Keuls test, compared to Propylene Glycol and Uninjected Controls.

EXPERIMENT 2. Prenatal Androgen Influences on the Organization of Feminine Sexual Behavior.

INTRODUCTION

Postnatal endogenous androgen is capable of defeminizing males while treatment with exogenous androgen postnatally can defeminize female rats (Ward, 1974; Whalen, 1974). However, the consequences of prenatal endogenous androgen on defeminization are laregly unknown. If androgen occuring prenatally acts similarly to that of postnatal androgen in inhibiting the organization of female sexual behavior, then prenatal treatment with the anti-androgen flutamide should block androgen-induced defeminization and facilitate the potential to display lordosis. Even though males are assumed to be undergoing the greatest amount of defeminization, by virtue of their active fetal testes, influences in the female cannot be assumed to be nonexistent, especially since Experiment 1 demonstrated the masculinizing presence of prenatal androgen. Thus, the prenatal influence of androgen on defeminization was assessed in males and females by administering flutamide to pregnant mothers and testing offspring for the display of lordosis in adulthood. A variety of estrogen dosages were used in this experiment to ascertain any adult estrogen-sensitivity enhancement induced by blocking defeminization prenatally.

METHOD

Treatment of pregnant females and the later testing of offspring for lordosis are described earlier in the General Methods section.

RESULTS

Effects of Prenatal Anti-androgen Treatment on Lordosis in Males

Flutamide treated males showed higher lordosis quotients than controls at the 2 ug ($F=4.44$, 2/22, $P < 0.025$) and at the 10 ug EB progesterone tests ($F=15.19$, 2/22, $P < 0.001$). There was a dose-response influence of flutamide on lordosis quotients with the higher dose (SCH 5) males showing significantly greater LQ's than either the lower dose of flutamide (SCH 1) or controls (SNK, $P < 0.05$) in the third test at each EB dosage (Figure 3).

When tested for the display of lordosis in response to estrogen alone (tests without progesterone) males that had been treated with flutamide prenatally showed significantly higher lordosis quotients than controls at both the 2 ug and 10 ug EB dosages ($F=15.69$, 2/22, $P < 0.001$ and $F=16.36$, 2/22, $P < 0.001$, respectively; Figure 4). Males prenatally exposed to the higher dose of flutamide (SCH 5) displayed significantly higher levels of lordosis over all tests at 10 ug EB than either the low dose flutamide group (SCH 1) or controls (SNK, $P < 0.05$).

There was no overall effect of progesterone augmentation of lordosis in males given either 2 or 10 ug EB in adulthood. Lordosis frequency of males in all three prenatal treatment groups was not significantly different (t-test) when pooled scores from the estrogen only test were compared with scores from the estrogen plus progesterone tests (Figure 5). However, during the second test at 10 ug EB, SCH 5 males scored significantly higher LQ's with the addition of progesterone in adulthood than they did under the same conditions without progesterone ($t=2.80$, 4, $P=0.05$). Similarly, SCH 1 males scored

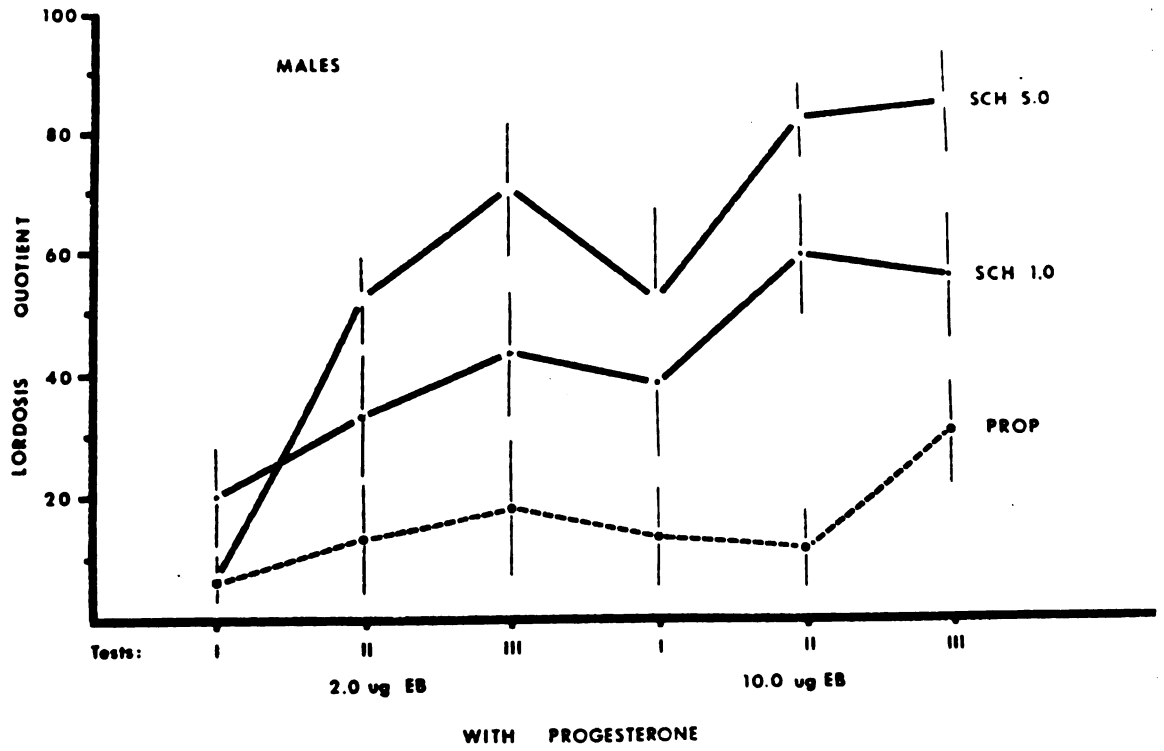

Figure 3. Mean lordosis quotients in castrated male rats treated with flutamide prenatally and given 2 ug or 10 ug EB and 500 ug progesterone as adults.

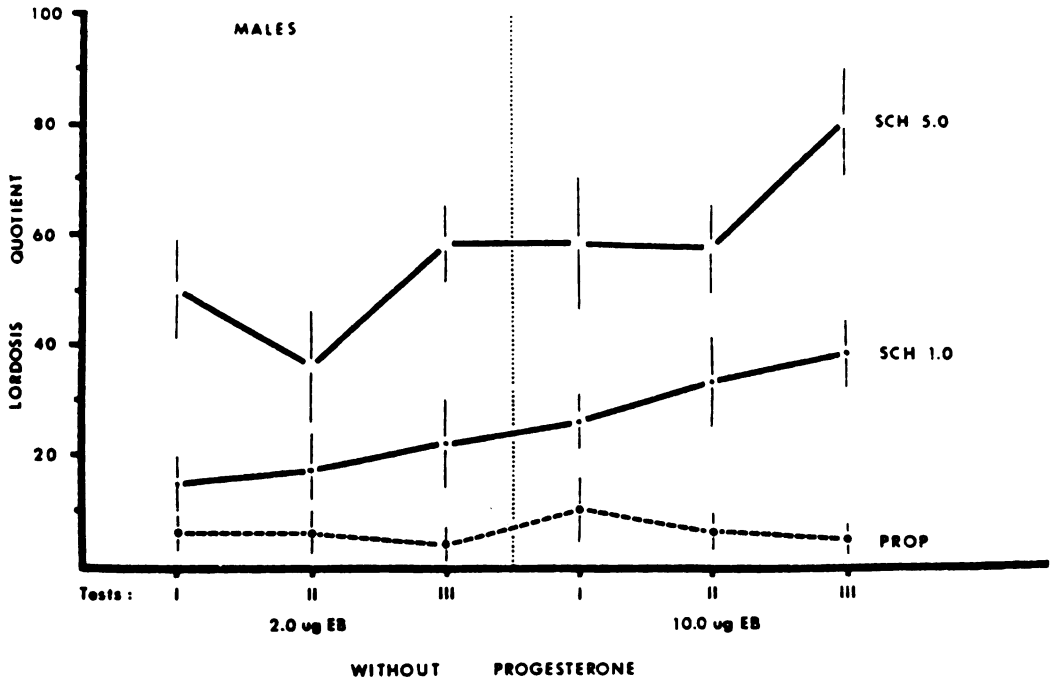

Figure 4. Mean lordosis quotients in castrated male rats treated with flutamide prenatally and tested as adults with 2 ug or 10 ug EB (no progesterone).

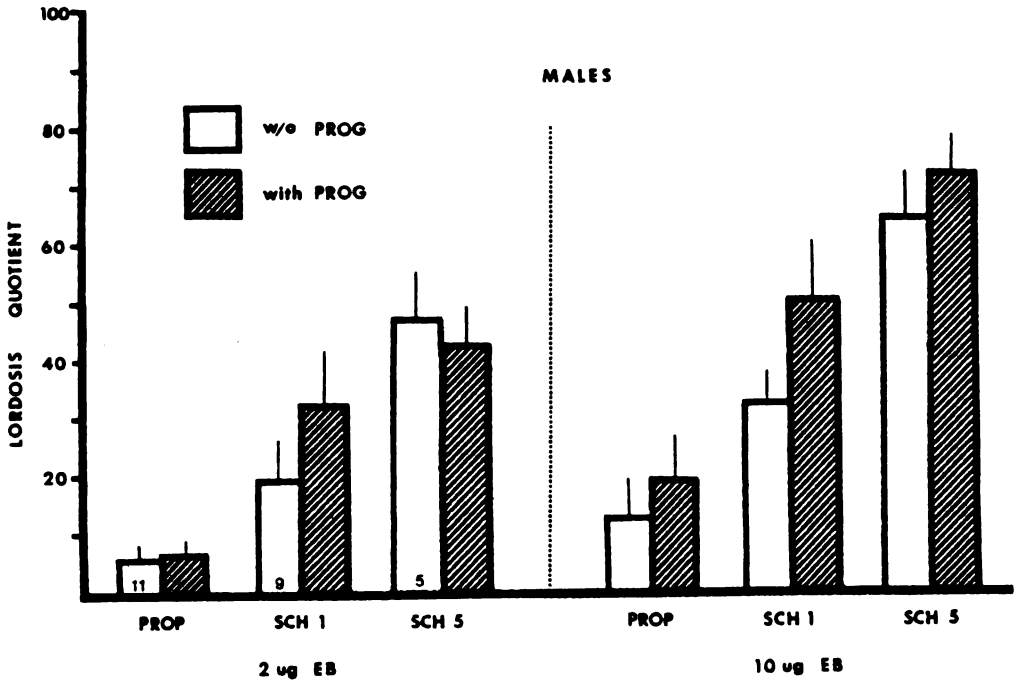

Figure 5. Comparison of mean lordosis quotients of castrated male rats treated prenatally with either flutamide or vehicle and tested in adulthood with either EB and Progesterone (with PROG) or EB alone (w/o PROG).

significantly higher lordosis quotients during the same test and dosage of EB when treated with progesterone compared to the test at that dose of EB without progesterone ($t=3.12$, 8, $P=0.014$).

Proceptive behavior was not observed in any of the male tests.

Influence of Prenatal Flutamide Treatment on Lordosis in Females

Tests with EB and Progesterone:

Female rats prenatally exposed to the higher dosage of flutamide showed higher lordosis quotients at the 0.175 ug, 0.25 ug and 0.5 ug dosages of EB than did controls ($F=4.54$, 2/26, $P < 0.02$, $F=15.31$, 2/26, $P < 0.001$ and $F=7.63$, 2/26, $P < 0.005$ respectively; Figure 6). At the 0.25 ug EB level animals exposed to either dose of flutamide did not differ from each other, yet both had greater lordosis quotients than control animals (SNK, $P < 0.05$). However, at the 0.175 ug EB level, females exposed to SCH 5 prenatally had significantly greater LQ's than females exposed to SCH 1 (SNK, $P < 0.05$). Lordosis quotients for females in all three treatment groups were very low at the 0.1 ug EB dosage and did not significantly differ from one another ($F=0.15$, 2/26, $P=0.85$). At the 1.0 and 2.0 ug EB dosages, lordosis quotients were very high for all groups and did not differ significantly from one another ($F=2.49$, 2/26, $P=0.103$, $F=1.17$, 2/26, $P=0.11$ and $F=2.52$, 2/26, $P=0.13$ respectively).

Tests with EB alone:

Females prenatally exposed to flutamide displayed significantly higher lordosis quotients than control females at 1.0 ug EB ($F=3.973$, 2/26, $P < 0.031$), 2.0 ug EB ($F=12.293$, 2/26, $P < 0.001$) and 10 ug EB

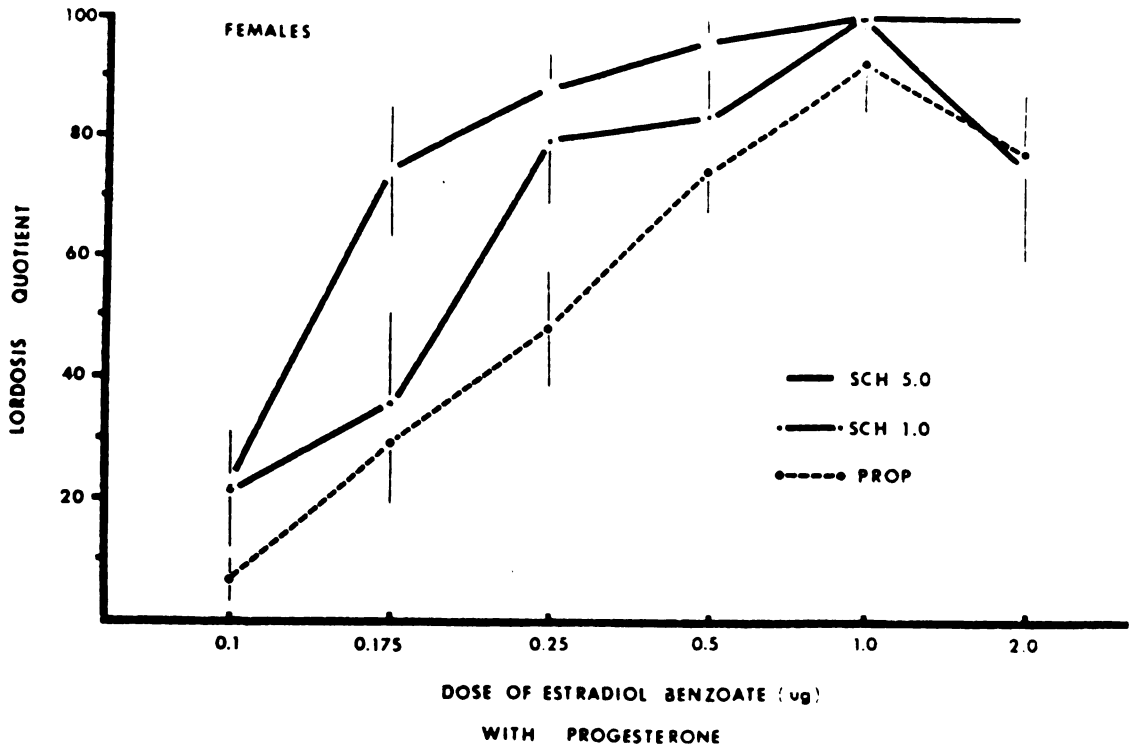

Figure 6. Mean lordosis quotients of female rats treated prenatally with either flutamide or vehicle and tested in adulthood at various dosages of EB with progesterone.

levels ($F=19.75$, 2/26, $P < 0.001$) in tests without progesterone. Animals exposed to the higher dose of flutamide (SCH 5) *in utero* showed significantly greater lordosis quotients than females in the lower flutamide group (SCH 1)(SNK, $P < 0.05$) at the 1 ug, 2 ug and 10 ug EB levels (Figure 7).

When the data for females are compared at tests in which 2 ug EB was given with or without the addition of progesterone, a significant effect of progesterone in facilitating the display of lordosis was seen (Figure 8). Unlike males, females in the control (PROP) and low dose flutamide (SCH 1) groups displayed significantly greater LQ's with progesterone than without (paired t-tests: $t=3.411$, 9 and $t=2.881$, 6; $P < 0.001$ and $P < 0.05$, respectively). The facilitation by progesterone is not seen in females exposed to SCH 5 presumably since those females are already at a high state of sexual receptivity without the addition of progesterone. Similar relationships between treatment with and without progesterone were seen at the 1.0 ug EB level as well (paired t-tests: $t=5.55$, 9 and $t=9.93$, 6 respectively; $P < 0.001$ for both comparisons).

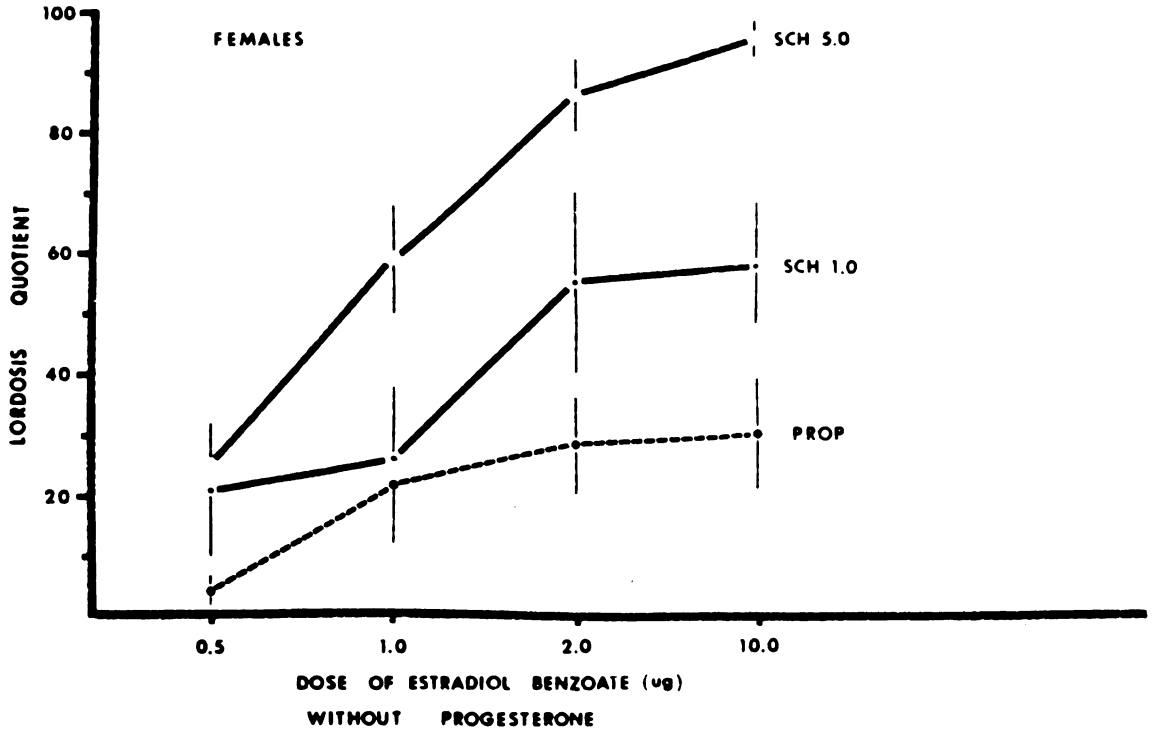

Figure 7. Mean lordosis quotients of female rats prenatally exposed to either flutamide or vehicle and tested in adulthood at various dosages of EB without progesterone.

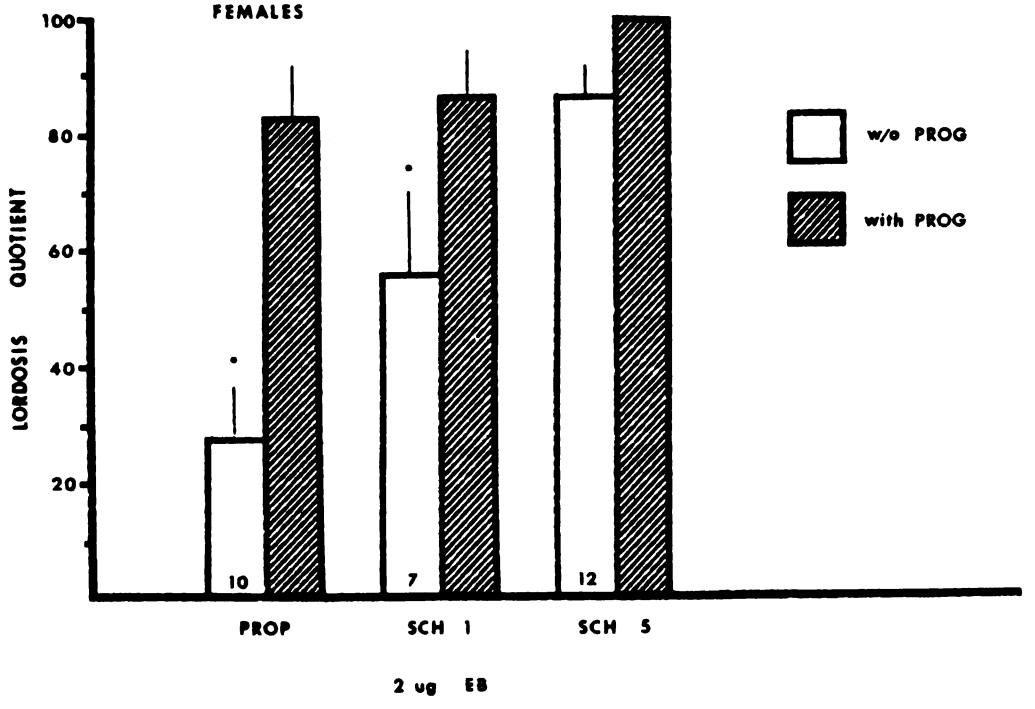

Figure 8. Comparison of mean lordosis quotients of female rats exposed prenatally to either flutamide or vehicle and tested in adulthood at 2 ug EB with P (with PROG) or EB alone (w/o PROG).

DISCUSSION

The differentiation of the potential for masculine and feminine sexual behavior in the rat begins prenatally under the hormonal influence of testosterone. This conclusion is based upon the present findings in which fetuses prenatally exposed to the anti-androgen flutamide showed decreased abilities to display adult masculine copulatory patterns in response to testosterone replacement in adulthood. Thus, masculinization, the organization of the potential for masculine copulatory behaviors via androgen action during development, is diminished by prenatal flutamide treatment, suggesting that this androgen-dependent process occurs prenatally and affects adult behavioral sensitivity to testosterone. Further, the ability of male and female rats to display feminine sexual behavior in response to ovarian hormones in adulthood can be enhanced by prenatally removing testosterone action with flutamide. Thus, flutamide is essentially blocking the process of defeminization thereby increasing the likelihood of an animal showing feminine sexual behavior as an adult.

While the concept that masculine sexual behavior in the female rat is dependent upon prenatal exposure to androgens is not new, earlier investigators did not effectively demonstrate whether changes in masculine copulatory behavior resulting from anti-androgen action on endogenous androgen influenced only peripheral genitalia or those central neural systems mediating mounting behavior (Nadler, 1969;

Ward, 1969). On the other hand, exposure to exogenous androgens during prenatal development has been shown to enhance the probability of mounting and intromission behavior (Gerall and Ward, 1966; Nadler,1969; Sodersten, 1973; Ward, 1974) indicating that the potential for androgens to influence neural systems mediating masculine sexual behavior existed during that period of development. Prenatal exposure to the anti-androgen cyproterone acetate (CA) has also been shown to reduce intromission responses or intromission-like responses in both male and female rats (Nadler, 1969; Ward, 1974; Ward and Renz, 1972) but mounting behavior was not affected.

While studies utilizing prenatal administration of testosterone report an increase of intromission frequency (Nadler, 1969; Ward, 1969; Ward and Renz, 1972; Whalen and Robertson, 1968; Whalen, Edwards, Luttge, and Robertson, 1969), increased frequency of non-intromittive mounts has not been a consistent finding of pre- or early postnatal treatment with testosterone (Whalen and Edwards, 1967; Whalen et al, 1969; Nadler, 1969; Ward and Renz, 1972). This failure to see a consistent increase in non-intromittive mounting in females as a result of administering testosterone perinatally, as well as the failure to see a decrease in mounting in males as a result of administering the anti-androgen CA, has led to the suggestion that androgen influences masculine sexual behavior largely by induction of phallus development (Beach, 1971; Nadler, 1969; Whalen et al, 1969; Whalen and Edwards, 1967). Increased phallic development then makes intromission more probable. Within the context of this argument, non-intromittive mounting is organized neurally in the absence of gonadal hormone influence.

The behavior of the untreated female rat cannot be taken as an indication of genetic potential for masculine sexual behavior in the absence of androgenic exposure. While the present findings lend support to the concept that intromission behavior has important androgenic determinants during development,quite possibly as a result of diminished penile development, these data also indicate that the mounting behavior of the normal female is not without benefit of prenatally occuring androgens. Reduced mounting in response to prenatal anti-androgen treatment indicates that mounting behavior in the female and male rat is initially organized, at least in part, by prenatal exposure to fetal androgens. Castration of newborn males then, removes a major portion of androgen influence, but since masculinization may have already begun prenatally, castrated males may still display some mounting behavior in adulthood, possibly accounting for the minimal levels of mounting observed in Day 1 (day of birth) castrated males.

The influence of the anti-androgen on masculinization parallels, in some respect, the effects of the steroidal anti-androgen CA. In Sprague-Dawley female rats the administration of 10 mg CA from days 10 to 22 of gestation decreased adult intromission frequency and non-intromittive mounting (Ward and Renz, 1972). In the present study 5 mg flutamide gave similar effects upon non-intromittive mounting. In these Long-Evans rats, however, the normal females showed such a low incidence of intromission patterns that the influence of flutamide upon this measure could not be evaluated.

Male offspring of mothers treated with 10 mg CA from days 13 to 22 of gestation failed to show changes in non-intromittive mounting, but intromission frequency was reduced (Nadler, 1969). In the present

study flutamide also reduced intromission frequency but failed to reduce non-intromittive mounting at both the 1 mg and 5 mg dosages. Paradoxically the 1 mg dosage of flutamide increased non-intromittive mounting in males. The reason for this increase is not clear, but might reflect a compensatory response to the lowered intromission frequency. The 5 mg dosage of flutamide may have blocked this compensatory response by a reduction in the copulatory rate. It is unlikely that such an increase in mounting in the 1 mg flutamide group would indicate an androgenic action of flutamide since experiments using flutamide to block testosterone-induced mounting in adult male and female rats showed no androgenic, and only anti-androgenic, activity of flutamide (Appendix, Experiment 2).

In addition to its effects upon frequency of intromissions and mounts, 5 mg flutamide also reduced the rate of achieving mounts and intromissions (MIPM). A similar reduction in copulatory rate has also been reported as a result of prenatal, but not postnatal, treatment with CA (Ward and Renz, 1972), and increased rates of mounting and intromission were also reported as a result of prenatal treatment with testosterone propionate (Ward and Renz, 1972). Together these reports strongly suggest a developing nervous system in which the potential to undergo masculinization exists prenatally. Temporal patterning of masculine sexual behavior apparently is not influenced by postnatal variations in androgenic stimulation (Schoelch-Krieger and Barfield, 1975). Rates of copulatory behavior in earlier prenatal studies were qualitatively reported, however, and may not accurately represent quantitative relationships to mounting, intromission and ejaculatory behavior patterns. In the present study, a quantification of the

temporal relationships of these events strongly indicated the prenatal developmental influences androgen has on organizing the overall pattern of masculine copulatory behavior.

It has been reported that flutamide might not influence the central behavioral actions of androgen since it did not block the effects of testosterone propionate on adult male sexual behavior in the rat (Sodersten, Gray, Damassa, Smith and Davidson, 1975; Gray, 1977). However, in the present series of experiments, flutamide was found to be highly effective in blocking the actions of testosterone on masculine sexual behavior in both male and female rats (Appendix, Experiment 2). Thus flutamide appears to have some central actions in the adult, and its action prenatally is most likely on the developing central nervous system tissues mediating the display of masculine sexual behavior. Additional experiments with flutamide in the adult failed to show any estrogenic or anti-estrogenic activities of this compound which might have influenced gestation or development (Appendix, Experiment 1). The lack of anti-hormonal actions other than specific anti-androgenicity makes flutamide a useful compound for studying prenatal developmental actions of androgens.

The importance of fetal androgens upon other aspects of masculine behavior and genital morphology in the female has recently been confirmed for the mouse (Gandelman, vom Saal and Reinisch, 1977; vom Saal and Bronson, 1978). Female mice which developed in proximity to male fetuses showed altered ano-genital distances similar to those reported earlier for rats (Clemens, 1974). In addition, female mice that develop next to male fetuses were found to have a shorter latency to fight (Gandelman, et al, 1977). Proximity to males <u>in utero</u> has also been

reported to decrease the overall sexual attractiveness and arousability of female mice compared to females situated further from the male (vom Saal and Bronson, 1978). These uterine proximity studies lend further support to the notion that normal female development takes place in an androgen environment and that removal of this androgen activity on their development diminishes the masculine component of the female's sexual behavior potential in adulthood.

While the influences of prenatal androgen on organizing masculine sexual behavior in both sexes were intriguing, particularly since they indicate that the display of mounting behavior in the normal female rat is influenced by hormone and not neccesarily an inherent capacity, such findings were not totally unexpected. However, the data obtained regarding prenatal androgenic influences on disrupting the potential for feminine sexual behavior were somewhat surprising. Males prenatally exposed to flutamide showed an enhanced lordosis in adulthood, presumably since their fetal testosterone was prevented from exerting its defeminizing action. However, feminine sexual behavior in female rats was also markedly facilitated by the administration of flutamide during prenatal development. This enhancement of the potential for female sexual behavior in both sexes seems to result from increased sensitivity to estrogen in adulthood.

This demonstration of increased lordosis frequency in females prenatally exposed to anti-androgen introduces the possibility that even normal female rats undergo some defeminization before birth, opening the argument that normal female rats are, in fact, not displaying their ultimate feminine sexual potential. Defeminization in rats can, of course, also be produced experimentally by postnatal treatment

with testosterone; this defeminization is also characterized by a decreased sensitivity to estrogen (Gerall and Kenney, 1970; Edwards and Thompson, 1970; Davidson, 1969; Hendricks, 1972) as well as a decreased progesterone sensitivity in adulthood (Clemens, Hiroi and Gorski, 1969; Sodersten, 1976). However, such experimental defeminization describes the postnatal potential for change in the female rat and does not address the issue of normality in feminine sexual behavior. It would appear that the normal female rat displays sexual behavior bisexually, with some masculine behavior potential existing, and yet complete feminine potential is limited by androgen exposure prenatally. Thus the variability of masculine and feminine sexual behavior in the female rat , and to some degree in the male as well, may be a function of both endogenous genetic variability regarding hormone sensitivity, and a more environmentally determined hormonally organized sensitivity to androgens and estrogens in adulthood.

The critical period for hormonal defeminizing capabilities has not been clearly defined. It seems that the time period wherein sensitivity to estrogen can be influenced perinatally ends sometime after Day 2 of life and before Day 5 (Edwards and Thompson, 1970; Pfaff and Zigmond, 1971). The onset of this critical period for the differentiation of estrogen-sensitivity seems to occur prenatally, but precise determination of onset is still unknown. Presumably, the maximum potential sensitivity to estrogen exists before fetal androgen secretion begins, at approximately day 14 of gestation. If androgen-influenced defeminization acts somehow by limiting adult capacity to respond behaviorally to estrogen, then this defeminization may begin at around the time of fetal androgen secretion. Treatment with flutamide, while beginning

before this secretory period, showed a dose response variability of effectiveness at blocking defeminization. SCH 5 treated females were less defeminized than SCH 1 treated females. Both the amount of androgen available to defeminize as well as the time available for its action to occur are most likely the key influences upon defeminization.

The reported differential effectiveness of pre-and postnatal androgen exposure on estrogen and progesterone responsiveness suggests that sexual differentiation of lordosis behavior may be divided into two seperate processes: a prenatal and postnatal phase in which competence to respond to estrogen develops and is partially suppressed by androgen, and a postnatal phase in which progesterone competence is established. In the normal female rat, responsiveness to progesterone develops postnatally in the absence of androgens, but in the normal male, progesterone responsiveness is suppressed by continued exposure to testicular androgens after birth, primarily from days 1 through 5 of life (Clemens et al, 1969; Clemens et al, 1970; Davidson and Levine, 1969; Beach, 1976).

This concept of sexual differentiation as a two-phase process is supported by the observation that the prenatal exposure of males to anti-androgen in this study resulted in an overall enhanced response to estrogen but not to progesterone. Since these males retained their testes until adulthood, it would be expected that they should not be sensitive to progesterone. Males castrated at birth, however, do respond behaviorally to progesterone in adulthood (Barraclough, 1967; Beach, 1971; Whalen and Edwards, 1967). That the testes were active postnatally in this study is further indicated by the lack of proceptive behavior in the males. Such behavior is though to develop in

the absence of testicular secretions postnatally (Beach,1976; Ward, 1974).

An alternative approach toward examining estrogen-sensitive and progesterone-sensitive aspects of sexual differentiation is suggested by recent reports concerning the role of estrogen in the development of progesterone receptors (Feder, Landau, Marrone and Walker, 1977; Kato, Onouichi and Okinaga, 1978; Leavitt, Chen and Allen, 1977; MacLusky and McEwen ,1978). Feder et al (1977) report that estrogen priming in female guinea pigs may increase the concentration of progesterone receptors, which are presumably involved in the facilitation of lordosis by progesterone. If estrogen enhances the number of progesterone receptors in the adult rat and if the frequency of these receptors is related to progesterone facilitation of lordosis, then any decrease in progesterone responsiveness as a function of perinatal treatment may reflect an alteration in progesterone sensitivity which is secondary to alterations in estrogen-dependent induction of progesterone receptors.

The failure of males prenatally exposed to flutamide to respond behaviorally to progesterone in adulthood does not exclude the possibility that some alteration in progesterone sensitivity might occur prenatally. While no overall significant differences were found between males given EB alone or EB with progesterone, on one test males exposed to either dose of flutamide prenatally did show a facilitation of lordosis when given P in adulthood, suggesting a possibility that progesterone-responsiveness could be enhanced by more potent prenatal anti-hormone treatments.

CONCLUSIONS

With the discovery that observed sexual dimorphisms in behavior began as a result of perinatal androgen activity on differentiation (Beach, 1942, 1971; Phoenix et al,1959; Harris and Levine, 1965), many investigators have sought to clarify the hormonal and temporal relationships influencing sexual differentiation. With the recent findings that development occurs both prenatally as well as postnatally, temporal influences on differentiation are beginning to be understood. However, the problem of hormonal interactions over time still precludes a total understanding of such a basic element of behavioral differentiation. The notion exists that while masculinization and defeminization are androgen-influenced processes, they may not neccesarily occur simultaneously or require similar hormone concentrations for their occurence.

In a review, Whalen (1974) presented data showing that, in the hamster, more androgen was required for defeminization when given postnatally than was required to effect masculinization, suggesting an independence of these two behavioral systems from one another regarding hormone sensitivity for their occurence. The present study, to some degree, addresses the issue of temporal and hormonal independence of masculinization and feminization in the rat. Qualitatively, a given amount of prenatally administered flutamide markedly influenced feminization but had less severe effects on blocking masculinization. If less hormone is required for masculinization, then the failure of

flutamide to totally block this behavior from developing may reflect an inability of the anti-androgen to interfere with all available testosterone. Effects of flutamide on defeminization were more noticeable, possibly because more of the defeminizing testosterone was prevented from acting, leaving less hormone to defeminize, but quite possibly enough to institute some masculinization.

It should be re-emphasized that the present study examined prenatal influences of an anti-androgen on males who were postnatally intact (retained their testes) until adulthood. Given that postnatal testosterone has potent masculinizing influences, effects of flutamide given to such males prenatally demonstrates considerable prenatal masculinization and developmental adaptability of the nervous system; an adaptability that may allow for substantial masculinization to be blocked prenatally by anti-androgens, and still show such a demasculinization despite considerable exposure to postnatal androgen.

An alternative interpretation of the findings reported here is that defeminization and feminization, as processes, may not only begin, but may primarily occur prenatally. If the _de facto_ behavioral potential of a developing fetus, regardless of its chromosomal sex, is feminine, then, before a male fetus may shift this organization toward a masculine pattern, it may initially require defeminization of the neural substrate before masculinization begins. The early secretion of androgen in the male starts this process and is then largely completed after birth by additional testicular secretions. The female, on the other hand, devoid of major internal androgen secretions prenatally, is only partially defeminized and masculinized be neighboring male testicular secretions _in utero_. Thus, feminization and masculinization

are prenatal processes in the female. No postnatal differentiation normally occurs in the female, although the potential for change is there, as demonstrated by numerous postnatal androgen experiments (reviewed in Beach, 1971).

Recently, a suggestion has been made that in the female sexual differentiation of feminine behavior and neuroendocrine secretory patterns may require estrogen to actively organize neural substrates, particularly the hypothalamus, that mediate feminine patterns of behavior and hormone secretion (Toran-Allerand, 1976). In this case, feminization is thought to be an active process requiring hormonal stimuli. Perhaps, then, defeminization preceding the organization of masculine sexuality may require excessive amounts of androgen to override or otherwise interfere with this type of active feminine behavior organization.

From a purely theoretical position, defeminization may need to occur before masculinization and, in this study, evidence exists to suggest that these behavioral organizations occur prenatally. In the male, the development of copulatory patterns may be analogous to morphological differentiation in which the dismantling of the female reproductive tract (Mullerian duct system) occurs before development of the male reproductive tract can proceed fully. Perhaps the organization of masculine copulatory behavior patterns first requires the removal of, or prevention against later development of, feminine behavior potential. In this regard, femininity may be the initial state of normality in the fetus, and masculinity is an alteration of the presumptive sexual status of the developing animal.

APPENDIX

APPENDIX

EXPERIMENT 1. Flutamide Action on Hormonally-Induced Feminine Sexual Behavior: Assessment of Behavioral Estrogenic and Anti-Estrogen Activity.

INTRODUCTION

The objective of this experiment was to determine whether flutamide had a direct facilitatory or inhibitory influence upon estrogen-induced lordosis. The concern regarding estrogen activity of this compound was based on the documentation that other anti-androgen compounds, particularly cyproterone acetate (CA), have a potent capacity to reduce sexual receptivity in ovariectomized female rats treated with estrogen and progesterone (Luttge et al, 1975). Apparently, CA , equally as potent as flutamide in interfering with androgen expression on androgen-dependent tissues, also has powerful estrogenic and anti-estrogenic activity. Possible antagonisms or enhancement by flutamide of estrogen-progesterone induced lordosis was therefore evaluated.

METHOD

Sixteen ovariectomized female rats known to be responsive to EB and P (based on lordosis) were assigned to a Latin Square Design for repeated measures to each of the following treatment conditions: (1) EB + PROP; (2) EB + SCH; (3) OIL + PROP; (4) OIL + SCH. The animals were tested once per week for four weeks for the display of

lordosis. Each week animals received a treatment condition different than that of the previous week according to their placement within the Latin Square. Thus each animal was tested under each treatment condition over the four week period. At the end of this period, another four week period of testing began using a different dosage of EB. The first four week period (Series 1) involved the administration of 0.5 ug EB or OIL given 48 and 24 hrs before testing with the addition of P (500 ug) 4 hrs before the start of testing. The second four week period (Series 2) was seperated from Series 1 by two weeks and involved the administration of a lower dosage of EB (0.25 ug) 48 and 24 hrs before testing with the addition of P (500 ug) 4 hrs before the onset of testing.

Flutamide (5 mg) or PROP was given concurrently with EB, 48 and 24 hrs before testing. The same dose of flutamide (SCH) was used in both series.

Data were analyzed for each series using the analysis of variance for Latin Squares with repeated measures, according to Kirk (1968). Mean comparisons between groups were made using a Student-Newman-Keuls test (SNK: Nie et al,1975; Winer, 1971).

RESULTS

Flutamide did not interfere with estrogen-progesterone induced sexual receptivity at either dosage of EB tested (Table 4). At the 0.5 ug EB dosage, an over all significant effect of treatment condition was observed ($F=605.16$, 3/48, $P < 0.0001$) but no significance was found among animals or over repeated testing ($F=2.437$, 3/48, $P < 0.06$ and $F= 0.840$, 3/48, $P < 0.5$, respectively). Treatment effects were wholly accounted for by differences between EB-treated and OIL-treated animals

Table 4. Mean Lordosis Quotients in Females Primed with either Estradiol Benzoate (EB) or Oil and Administered either Flutamide (SCH) or Propylene Glycol (PROP).

Treatment *	0.25 ug EB	0.5 ug EB
EB + PROP	62.50 ± 6.61	90.00 ± 2.42
EB + SCH	76.88 ± 7.34	88.12 ± 3.32
OIL + PROP	3.40 ± 1.19	3.13 ± 1.20
OIL + SCH	3.00 ± 1.00	3.13 ± 1.19

* n=16, all animals received all treatments in a Latin Square repeated measures design.

All values given as Mean S.E.M.

(SNK, $P < 0.01$). No significant differences between EB + PROP and EB + SCH treatment groups were found (SNK, $P > 0.1$). Table 4 reveals that at either dose of EB mean lordosis quotients for EB + PROP and EB + SCH conditions are virtually identical.

At the 0.25 ug EB dosage similar results were obtained with an overall treatment effect ($F=61.654$, 3/48, $P < 0.0001$) observed. No significant differences between EB + PROP and EB + SCH or between OIL + PROP and OIL + SCH groups were observed. Treatment effects were wholly accounted for by EB vs. OIL group comparisons (SNK, $P < 0.01$).

DISCUSSION

The possibility that flutamide might have subtle estrogenic actions was not supported since lordosis behavior, a sensitive behavioral indicator of estrogen activity, of females treated with OIL + SCH did not significantly differ from LQ's obtained in animals treated with OIL + PROP, with both levels of lordosis being quite low. Thus flutamide at doses equivalent to those used in assessing CA activity on lordosis, neither facilitated nor inhibited lordosis in female rats.

This experiment clearly demonstrates that flutamide does not exert an estrogenic or anti-estrogenic influence on behavior per se. The interpretations of flutamide actions in adults and neonates may thus rule out any strong indication that this anti-androgen directly interferes with estrogenic expression.

EXPERIMENT 2. Flutamide Action on Testosterone-Induced Masculine Sexual Behavior in Male and Female Rats.

INTRODUCTION

Flutamide, while exerting potent anti-androgenic actions on peripheral target tissues and centrally mediated hormonally organized patterns of behavior, appears to have neither estrogenic nor anti-estrogenic effects upon lordosis (Appendix A, Experiment 1). Although the demasculinizing influences of flutamide have been interpreted as a result of both central neural actions as well as actions upon genital morphology,results of studies on adult rats may cause a questioning of the extent of flutamide influence on central neural mechanisms. Studies reported by Sodersten et al (1975) and Gray (1977) have suggested that flutamide exerts powerful anti-androgenic actions upon peripheral androgen-dependent tissues such as the penis and seminal vesicles, but may exert no central actions upon mounting or intromission behavior in the adult.

The present experiment examined the anti-androgenic ability of flutamide to block the re-initiation of testosterone-induced mounting and intromission behavior in males who had not achieved a mount within several weeks after castration. In this way, it was hoped that any anti-androgenic actions flutamide might have on blocking adult sexual behavior in males would be maximally observed. Additionally, female rats, which show little or no mounting behavior as adults, were used as models to test for the ability of flutamide to block the initiation of mounting behavior resulting from testosterone treatment as adults. Further, since other anti-androgens, such as CA, have shown androgenic side effects

(Neumann and Steinbeck, 1974) possible androgenic actions of flutamide on masculine sexual behavior were considered. Thus, this experiment hoped to clarify some of the anti-hormonal characteristics of this novel non-steroidal anti-androgen (flutamide).

METHOD

Part 1: Effect of Flutamide on Masculine Sexual Behavior in Females.

Sixteen female rats were ovariectomized at approximately 85 days of age, allowed a two-week post-surgical recuperation phase and then tested for the display of masculine sexual behavior as described earlier in Prenatal Experiment 1. All females received two post-surgical tests to determine the presence of masculine behavior potential. None of the females mounted on either test. Experimental females were then randomly assigned to one of two treatment groups: a) flutamide (SCH) 5 mg/day + testosterone (T), 100 ug/day, n=8; b) propylene glycol (PROP) + T, n=8. All injections were given simultaneously in a volume of 0.1 ml. Animals were then tested for the display of masculine sexual behavior once a week for five weeks. After this five-week period, treatments were reversed for both groups and an additional five weeks of testing was administered. This latter procedure was employed to ascertain any possible effects of flutamide on blocking the maintenance of copulation.

Part 2: Effect of Flutamide on Masculine Sexual Behavior in Males.

Twenty four male rats known to be proven copulators were used in this experiment. Males were castrated at approximately 110 days of age. Two months subsequent to castration males were tested once a week for the display of masculine sexual behavior until they failed to show any

mounting behavior on three consecutive tests. At this point animals were randomly assigned to either: a) testosterone (T, 100 ug/day) + flutamide (SCH, 5 mg/day), n=12; or b) T + PROP, n=12. Each test for masculine sexual behavior followed the format for males described earlier (Prenatal Experiment 1). Males were tested weekly for eight (8) weeks after which time the dosage of flutamide was increased to 10 mg/day while the testosterone dose was held constant at 100 ug/day. Tests then continued for an additional eight weeks at this higher dose of flutamide. Upon completion of the experiment animals were sacrificed, body weight, ano-genital distance and penile diameter recorded, and their seminal vesicles and ventral prostate removed for weighing. The penis of each remaining animal was also removed for histological examination.

RESULTS

Part 1: Effect of Flutamide on Masculine Sexual Behavior in Females.

Testosterone-treated female rats given concurrent flutamide treatment displayed significantly lower frequencies of mounting behavior than did T + PROP controls (Figure 9). This effect was most apparent by the fifth weekly behavior test ($F=7.58$, 1/14, $P < 0.01$). In addition, the copulatory rate (MIPM) of T + SCH females was significantly lower than that of controls (0.106 ± 0.10 and 0.725 ± 0.21 MIPM, respectively ($F=6.83$, 1/14, $P < 0.02$). No other statistically significant differences with regard to mount frequency or MIPM were found at earlier tests. For those tests in which treatments were reversed, no statistically significant differences between groups were found, indicating that flutamide treatment failed to block mounting behavior proceeding at an already

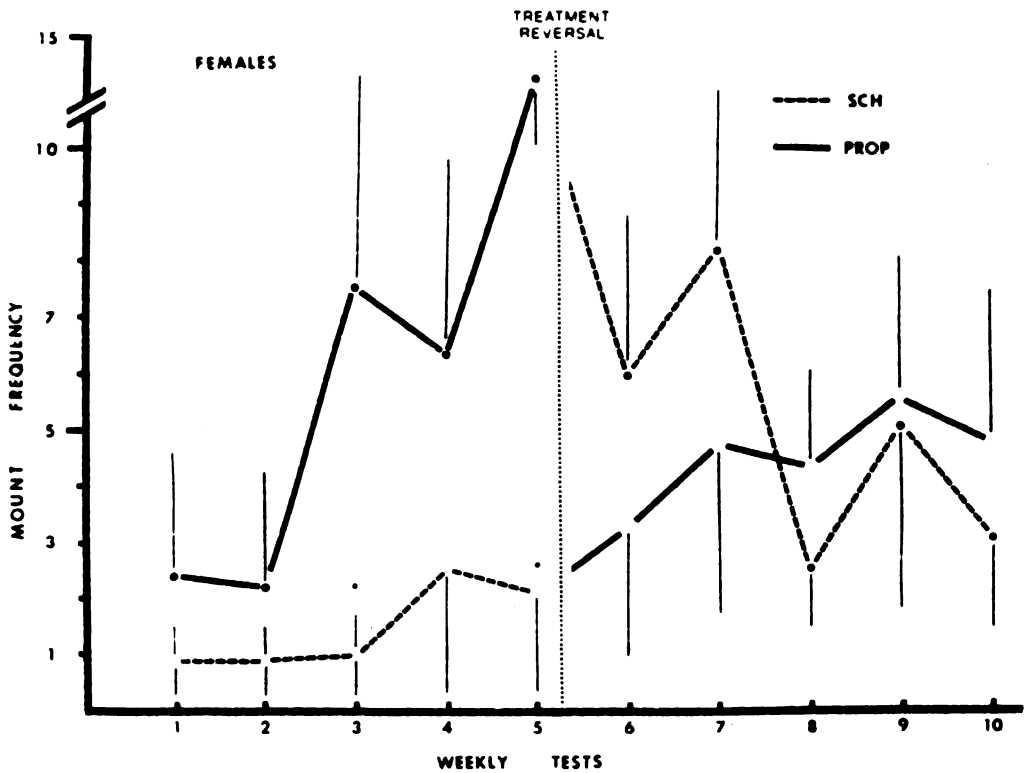

Figure 9. Mean mount frequency in female rats treated as adults with either flutamide (SCH) or vehicle (PROP) in combination with 100 ug testosterone. Tests 6 through 10 involve treatment reversal (see text) and are indicated by switched lines.

high rate in testosterone-treated females.

The percentage of flutamide-treated females which mounted was significantly lower than that of controls, on both the fourth and fifth behavior tests (Fisher's Test: $P < 0.05$ and $P < 0.01$, respectively). No significant differences were found between groups at any time in post-reversal tests (Figure 10).

Part 2: Effect of Flutamide on Masculine Sexual Behavior in Males.

The frequency of intromission behavior was significantly reduced in testosterone-treated males exposed to either 5 mg flutamide ($F=5.03$, 1/21, $P < 0.036$) or later at 10 mg flutamide ($F=8.73$, 1/16, $P < 0.009$) (Table 5). The percentage of animals displaying intromission pattern was also significantly reduced by treatment with flutamide at either dose (Fisher's Test, $P < 0.01$). Ejaculation was not shown by animals receiving flutamide. The percentage of animals that achieved ejaculation was significantly greater than flutamide treated males at either 5 mg ($P < 0.01$) or 10 mg dosages ($P < 0.01$, Fisher's Test).

The percentage of males showing mounting behavior was significantly greater for controls than for flutamide treated males at the 10 mg dose ($P < 0.05$, Fisher's Test)(Table 5). While the frequency of mounts was not significantly affected by treatment with 10 mg flutamide ($F=0.406$, 1/16, $P=0.53$) the copulatory rate (MIPM) was significantly lower in flutamide treated males than in controls ($F=4.34$, 1/16, $P < 0.05$). Copulatory rate was also significantly lower for flutamide treated males than for controls at the 5 mg dose of flutamide ($F=3.75$, 1/21, $P < 0.05$).

The percentage of males showing mounting behavior did not significantly differ between groups during any test in which 100 ug T and 5 mg

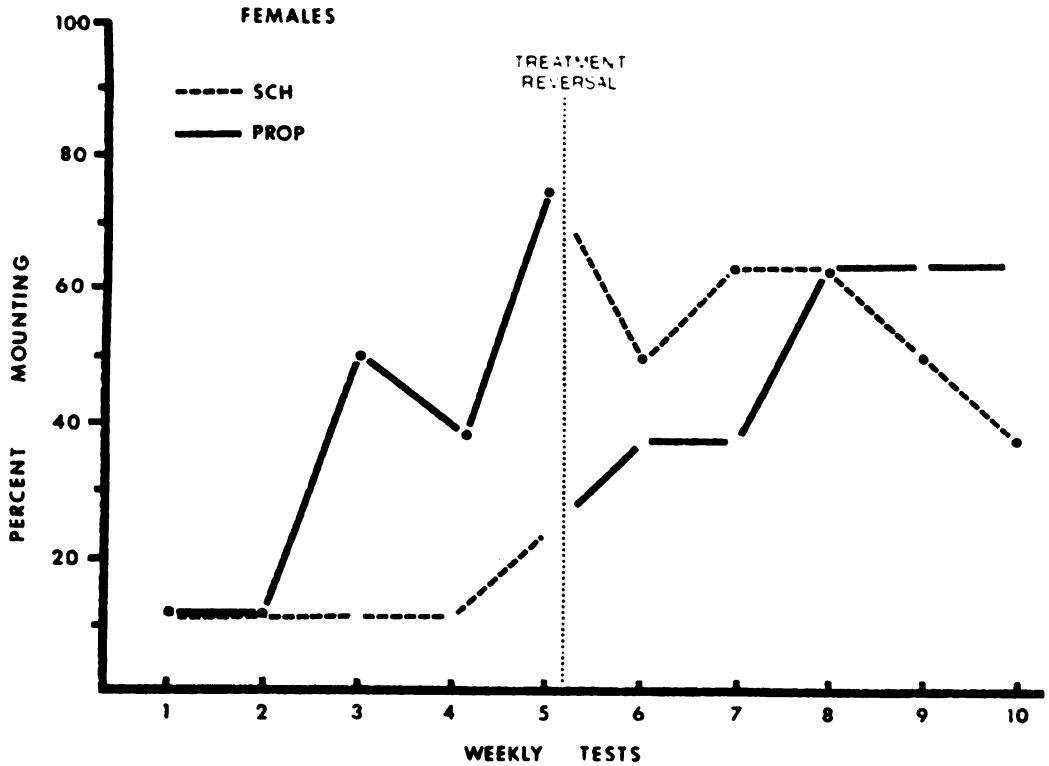

Figure 10. Percentage of female rats mounting. Animals received either flutamide or vehicle in combination with 100 ug testosterone. Reversed group lines indicate treatment reversal (see text).

Table 5. Masculine Sexual Behavior in Long-Term Castrated Male Rats Given Testosterone (100 ug) Daily for 16 Weeks with Flutamide (5 mg) given for the first 8 Weeks then Flutamide (10 mg) for the second 8 Weeks.

	Controls	Flutamide(5 mg)	Controls	Flutamide(10mg)
% Mounting	50	33	100**	45
Mount Frequency	7.70 ± 2.80	3.40 ± 2.10	8.60 ± 1.80	6.20 ± 3.40
% Intromitting	45**	0	66**	0
Intromission Frequency	1.45 ± 0.60*	0.0	9.80 ± 3.20*	0.0
% Ejaculating	36**	0	60**	0
MIPM	0.66 ± 0.23*	0.17 ± 0.10	1.22 ± 0.38*	0.40 ± 0.22
Number of animals	11	12	10	10

All values given as either percents or Mean S.E.M.

* P < 0.05, Analysis of Variance

** P < 0.05, Fisher's Probability Test

MIPM= Mounts and Intromissions per Minute.

flutamide were administered, nor did overall mount frequency differ from that of controls ($F=2.23$, 1/21, $P=0.15$).

Seminal vesicle weight was significantly reduced in flutamide-treated castrate males concurrently receiving testosterone replacement therapy ($F=29.271$, 1/16, $P < 0.001$). Ventral prostate weight was also significantly reduced in flutamide-treated males compared to controls ($F=3.612$, 1/16, $P < 0.001$)(Table 6). Males treated with flutamide had significantly fewer cornified papillae (penile spines) of the glans penis than controls ($F=83.69$, 1/16, $P < 0.001$) as well as having penes of smaller diameter ($F=33.74$, 1/16, $P < 0.001$). No significant differences between treatment groups were found for body weight or anogenital distance (Table 6).

DISCUSSION

Masculine sexual behavior in testosterone-treated male and female rats was diminished by adult treatment with flutamide. The degree of this decrease in masculine copulatory behavior was pronounced with regard to intromission and ejaculatory behavior in males, and mounting and MIPM in females. Mounting behavior was significantly decreased in female rats after five weeks of a 50:1 ratio of flutamide (5 mg/day) to testosterone (100 ug/day) treatment. In male rats, however, the 50:1 treatment did not affect mounting behavior in experimental animals when compared to control animals. These findings for the male are similar to those reported by Sodersten et al (1975) and Gray (1977). However, when the dose of flutamide in males was increased to a 100:1 ratio, decreases in mounting behavior were observed. The percentage of males displaying mounting behavior and their overall rate of copulation was significantly

Table 6. Effects of Long-Term Treatment of Male Rats with Free Testosterone (T; 100 ug) and the Anti-Androgen Flutamide on Certain Reproductive Tissues and Body Morphology.

	Flutamide + T	Controls (PROP + T)
Body Weight (gms)	523 ± 17	511 ± 17
Ano-Genital Distance (mm)	44.5 ± 1.2	45.0 ± 1.0
Penile Diameter (mm)	4.04 ± 0.08 **	4.90 ± 0.12
Seminal Vesicle Weight (mgs)	150.5 ± 10.3 **	255.1 ± 16.3
Ventral Prostate Weight (mgs)	223.7 ± 18.4 *	275.2 ± 20.4
Number Cornified Papillae	22.1 ± 2.4 *	45.3 ± 1.2
Number of Animals	9	9

All values other than Number of Animals given as Mean S.E.M.

* significantly different from Controls, $P < 0.05$

**significantly different from Controls, $P < 0.01$

lower in flutamide-treated animals than in controls (Table 5). Thus, it would appear that the potential for mounting is greater in males than in females, but the occurence of mounting in either sex is a testosterone dependent response.

The display of intromission patterns was significantly reduced in males receiving flutamide treatment at either dosage. All measures of intromissive and ejaculatory behavior were significantly reduced in males treated with flutamide compared to control animals. Changes in ejaculatory behavior reported here are consistent with previous studies using flutamide (Sodersten et al,1975; Gray, 1977). These investigators, however, failed to find an effect of flutamide on intromission frequency presumably since they were testing animals that had not lost intromission behavior at the start of their test sequence. The ability of androgen to restore intromission behavior and mounting is related to the degree to which these behaviors are qualitatively and quantitatively present in the animal at the time of androgen replacement. In this study, flutamide was capable of blocking intromission, and to some degree, mounting, possibly because the dose of anti-androgen to androgen was greater than for these other workers, and also possibly because animals in the present study had lost all copulatory behavior prior to treatment with androgen and flutamide.

Thus it would appear that flutamide is capable of exerting powerful central and peripheral anti-androgenic activity without contributing any discernable androgenic side effects.

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" If I have seen farther, it is because I have
stood on the shoulders of giants."

- attributed to Isaac Newton

" It is not enough to see farther by standing
on the shoulders of giants;
One must also know in which direction to look"

- attributed to Brian Gladue