ELSEVIER

Physiology & Behavior 79 (2003) 633–641

PHYSIOLOGY & BEHAVIOR

Play, copulation, anatomy, and testosterone in gonadally intact male rats prenatally exposed to flutamide

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Received 26 September 2002; received in revised form 13 December 2002; accepted 29 April 2003

Abstract

The role of prenatal androgen on the differentiation of sexually dimorphic juvenile play and adult copulatory patterns was evaluated in male offspring of rats injected with 5 mg of the androgen receptor blocker flutamide (4′-nitro-3′-trifluoromethylisobutyranilide) from Days 11–21 of pregnancy. Rough-and-tumble play was incompletely masculinized in flutamide-exposed males at 31 days of age. The copulatory potential tested at 70 days of age was severely attenuated by prenatal flutamide. There was no ejaculatory behavior, low levels of intromissions, and depressed levels of nonintromittive mounting when the animals were tested while gonadally intact. Adult plasma levels of testosterone (T) were not different in flutamide-exposed males and controls, but testicular and epididymal weight, anogenital (AG) distance, and penile length were reduced. While reductions in intromittive mounting and ejaculatory behavior may be due to the abnormalities in the external genitalia, the incomplete masculinization of play and the reduction in nonintromittive mounting probably resulted from effects the androgen antagonist exerted on sexual differentiation of the central nervous system. These data suggest that androgen released prior to birth is needed for the full masculinization of juvenile play behaviors in the rat, just as it is for the adult copulatory pattern.

Keywords: Fetal; Play fighting; Antiandrogen; Prepubertal

1. Introduction

In mammals, masculinization and defeminization of the reproductive system and behavioral potentials are induced by the action of testosterone (T) or its metabolites during certain critical periods of perinatal development. In rats, the "organizational" influences for copulatory behaviors are exerted during both a prenatal stage, the last week of gestation, and a postnatal period, the first few days after birth (e.g., see review by Ward and Ward [1]). In contrast, the existing dogma regarding the critical period for sex differences in rough-and-tumble play behaviors is that differentiation takes place exclusively during early postnatal development [2,3]. Supporting the importance of neonatal T exposure are studies showing that males that had been castrated shortly after birth show the low levels of play fighting that normally characterize females [4,5]. A major goal of the present study was to test the hypothesis that sexual differentiation of play, like copulatory patterns, begins during prenatal ontogeny. The effect of prenatal T exposure on sexual differentiation of play was assessed by blocking androgen receptors in developing males by exposing their dams to the drug flutamide (4′-nitro-3′-trifluoromethylisobutyranilide) during late pregnancy. Flutamide is a nonsteroidal androgen receptor antagonist that crosses the placental barrier and has little or no direct androgenic or estrogenic actions [6]. Males given flutamide during postnatal days 1–10 [7] show low levels of play fighting, but play behavior has not been assessed in males exposed to the antiandrogen during prenatal development.

Male rats prenatally exposed to flutamide have been reported either to have no deficits in their adult copulatory behavior [8] or to show deficits in some components of the pattern, such as intromissions and ejaculations, but not in nonintromittive mounting [9,10]. However, in all previous flutamide studies, males were castrated, either as neonates or as adults, and treated with pharmacological doses of testosterone propionate (TP) before behavioral testing. Our

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doi:10.1016/S0031-9384(03)00120-3

laboratory has previously demonstrated that testing under conditions of large doses of exogenous T [11,12] may obscure the behavioral effects of prenatal treatments that suppress T. These findings raise the question as to whether the mounting component of the male copulatory pattern was actually normalized in flutamide males, to the exclusion of intromissions and ejaculation, or whether abnormalities in mounting were masked by the methodology used to assess the behavior. One purpose of the present investigation was to evaluate whether males prenatally exposed to an antiandrogen also incur deficits in nonintromittive mounting. However, the abnormality might be revealed only when the animals are tested under the influence of normal endogenous T titers, i.e., while gonadally intact. Adult levels of plasma T were measured to rule out the possibility that behavioral abnormalities are linked to deficiencies in adult testicular activity resulting from the prenatal treatments.

2. Methods

2.1. Subjects

Nulliparous female Sprague–Dawley rats (Harlan Sprague–Dawley, Madison, WI) obtained at 50 days of age were time mated between 60 and 70 days of age. Thirty-one of these females produced litters yielding 354 pups that were used in various phases of this experiment. Throughout the study animals were maintained on a reverse day/night cycle (lights on 2300–1100 h) in a temperature-controlled vivarium. Food and water were available ad libitum. These studies adhered to the standards on animal care and treatment described by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23) and were approved by the Villanova University Animal Care and Use Committee.

2.2. Mating and rearing procedures

Estrous females were paired with males until two ejaculations had been observed (Day 0 of pregnancy). Pregnant females were housed individually in Plexiglas cages ($43 \times 23.5 \times 20$ cm) lined with hardwood chip bedding, and were given ad-libitum access to food and water.

Beginning on Day 11 and continuing through Day 21 of gestation, dams randomly assigned to the flutamide group ($n = 15$) received a daily subcutaneous injection of 5 mg of flutamide (Schering, Bloomfield, NJ), suspended in 0. 15 cc of propylene glycol. Control dams ($n = 20$) received an injection of the propylene glycol vehicle. On Day 21 of gestation, all dams were supplied with fresh bedding and nesting materials. Bedding was not changed again until Day 14 postpartum.

Day 14 was the first day on which pups were handled. On this day, control female pups were differentiated by the presence of mammillary buds, which were not apparent in control males. It was impossible to sex flutamide animals in this way, as all drug-treated animals possessed mammillary buds. Also on Day 14, the anogenital (AG) distance of all pups was measured with calipers and a metric ruler (to the nearest 0.5 mm), and each was weighed (to the nearest 0.1 g). The animals then were left undisturbed until weaning.

All animals were weaned, individually marked, and weighed at 21 days of age. Sixteen behavioral test groups were formed. Each was housed in a Plexiglas group cage ($46 \times 32 \times 20$ cm) lined with hardwood chip bedding. Each group consisted of 6 unrelated animals born within 24 h of each other. Each group was composed of 2 control males, 2 flutamide males, and 2 control females. Except for these constraints, the animals were randomly selected.

At the time of weaning, every animal in each test group was coded with blue ink. The code consisted of marking either the right or left ear, front limb, or hind limb. Animals were re-marked on the morning of the days on which play behavior was to be scored. The code was withheld from the experimenter scoring behavior so that the experimenter was blind to the sex and prenatal treatment of the animals.

2.3. Play behavior testing

Observations of rough-and-tumble play took place in the home cage when the animals were 27, 31, and 35 days old. These test days were chosen because they include the period when peak levels of play behavior occur in the Sprague–Dawley strain of rats housed under the conditions that prevail in our vivarium [13]. Approximately 20 h before the play behavior of a group of animals was to be scored, the home cage was moved into a curtained enclosure located within the vivarium. The day/night cycle in this enclosure was the same as in the rest of the vivarium. During the period of behavioral observation, animals were exposed to constant low levels of red illumination provided by two 25-W red incandescent lights.

Four groups of animals were scored at a time during the first 4 h of the dark cycle. A Datamyte 900 electronic data recorder (Electro General) was used to record the occurrence of play behaviors. The experimenter was located approximately 1 m in front of the home cages and observed each of the six focal pups in a group sequentially for 30 s. Once every pup had been observed, the experimenter scored the next group of animals and repeated this procedure until all four groups to be scored on that day had been completed. This entire procedure was repeated 15 times, so that each animal received fifteen 30-s observations, totaling 7.5 min per day. At the end of each observation day the animals were weighed.

Before each session, the order of observation of the four cages and the order in which animals within a cage were to be observed were randomly determined. The rough-and-tumble components of play fighting (i.e., pouncing, pinning, wrestling, boxing, and on-the-back) were recorded. The frequency of each component of play behavior exhibited

by the focal animal and the play partner toward whom the behavior was directed were scored separately. All data collected for a subject on a given day were summed. Operational definitions of the behaviors are as follows: (1) pouncing: one animal lunges at another animal with its forepaws extended; (2) wrestling: two animals tumble and roll over one another in a seeming attempt to gain a dominant position; (3) boxing: both animals rear up on their hind legs and make jabbing movements at each other with their forepaws; (4) pinning: the animal that is pinning stands over the opponent with its forepaws on the ventral surface of the opposing animal. This is considered a dominant posture; (5) on-the-back: this is the reciprocal behavior of pinning. When an animal is in this position the entire ventral surface is exposed. This is considered a submissive posture (for more detail, see Ref. [13]).

On Day 36, AG distances were measured for all offspring in the 31 litters.

2.4. Male copulatory behavior testing

At 40 days of age, all males that had been tested for juvenile play were rehoused as same treatment pairs in suspended cages ($34.5 \times 18 \times 18$ cm). At approximately 70 days of age, testing of all males for male sexual behavior began. At the beginning of each test, the animals were placed into individual semicircular observation arenas ($53 \times 33 \times 23$ cm) for 5 min. An ovariectomized estrous female was added for 30 min. Females were brought into estrus with injections of 10 μg estradiol benzoate and 0.5 mg progesterone, given 48 and 6 h, respectively, prior to testing. Mounts, mounts with intromissions, and ejaculations exhibited by the experimental animals were recorded on a Datamyte 900 electronic data recorder. This procedure was repeated at 3-day intervals until all males had received three tests.

Mount and intromission frequencies for each male were calculated with respect to "active time" during the three tests. Active time refers to the amount of test time during which the male was not in a postejaculatory state of inactivity. For animals that ejaculated, the lengths of all postejaculatory intervals (PEIs) were subtracted from the total test time. The use of "active time" in the calculation prevents deflation of mount and intromission frequencies due to ejaculations and ensuing PEIs.

2.5. Morphological measures

At approximately 120 days of age, body weight and AG distance were recorded for 10 flutamide females, 16 control females, 16 flutamide males, and 16 control males. The males were then anesthetized with pentobarbital and chloral hydrate. The penile sheath was retracted as far as possible and, using calipers and a ruler, the distance from the tip of the penis to the retracted sheath was measured. Also, the left testis and epididymis were removed, separated, and weighed individually. If for any reason the left testis or epididymis could not be used, the right organs were measured. Many flutamide males had a blind vaginal opening. The depth of this opening was measured with the use of a probe and metric ruler.

2.6. Radioimmunoassay (RIA)

At approximately 400 days of age male siblings of the animals used in behavioral testing (37 control males, 23 flutamide males) were stunned by a blow to the head, killed by decapitation, and their trunk blood was collected in EDTA-coated (20 nM) test tubes. The blood was kept at room temperature for 30 min and then was centrifuged for 5 min. Plasma was pipetted into a test tube and was stored at -60 °C. A radioimmunoassay for T was performed on duplicate samples. The antiserum for the T assay was provided by Dr. G.D. Niswender of Colorado State University. Testosterone levels were measured using previously described methods [14]. Chromatographic separation of androgens was not performed, as T levels do not differ between chromatographed and nonchromatographed samples in Sprague–Dawley rats [15]. Samples were extracted with anhydrous ethyl ether and were then incubated with the antiserum. The minimum sensitivity of the T assay was 10 pg/tube. The intra- and interassay coefficients of variation were 6.5% and 10%, respectively.

3. Results

Flutamide had no effect on the success of pregnancy. Thirteen of 15 flutamide-injected females gave birth compared to 18 of 20 of the control dams. There also were no apparent effects of flutamide on litter size measured on Day 14 postpartum (control litters averaged 11.9 pups vs. 11.7 in the flutamide litters), or on survival of the pups thereafter.

The sex of the flutamide-exposed pups could not be determined in many cases, when first examined on Day 14 postpartum. The external appearance of the genitalia and scrotal area was indistinguishable in males and females. Furthermore, all flutamide animals had mammillary buds. Control animals were sexed by the presence of mammillary buds in females and the absence of these buds in males. A one-way ANOVA on the litter means for AG distance revealed significant differences among flutamide animals (collapsed across sex), control males, and control females, $F(2,46) = 144.29$, $P < .0001$. Tukey HSD tests indicated that the mean AG distance of control males (8.6 ± 0.14 mm) was significantly ($P < .01$) larger than that of both control females (5.8 ± 0.14 mm) and flutamide animals (5.8 ± 0.10 mm). The latter two groups did not differ. The small standard error associated with the mean AG distance of the flutamide animals is especially remarkable, given that this group turned out to have an equal proportion of males and

females. There were no significant differences in body weight among flutamide animals (32.78 ± 1.14 g), control males (32.50 ± 0.82 g), and control females (31.17 ± 0.19 g). Thus, the short AG distance of flutamide exposed animals on Day 14 was not the result of a general decrease in body size.

By the time of weaning (Day 21), the sex of the flutamide exposed males could be accurately determined by palpation of the as yet undescended testes. A 2 × 2 (Sex × Prenatal Treatment) analysis of covariance was performed on litter means of Day 21 body weight with litter size included as a covariate. The effects of both sex and prenatal treatment were significant. As depicted in Fig. 1a, males weighed more than females, $F(1,55) = 7.53$, $P < .01$, and flutamide-exposed animals weighed more than controls, $F(1,55) = 5.32$, $P < .03$. The interaction between sex and prenatal treatment was not significant. The litter means of AG distance recorded on Day 36 are shown in Fig. 1b. A 2 × 2 (Sex × Prenatal Treatment) ANOVA showed that males had a significantly longer AG distance than females, $F(1,56) = 1108.17$, $P < .0001$, and controls had a longer AG distance than flutamide-exposed animals, $F(1,56) = 162.40$, $P < .0001$. The interaction between sex and treatment was also significant, $F(1,56) = 117.36$, $P < .0001$. T tests revealed that control males had a significantly longer AG distance than flutamide males, $t(28) = 199.60$, $P < .0001$, but that control and flutamide females did not differ.

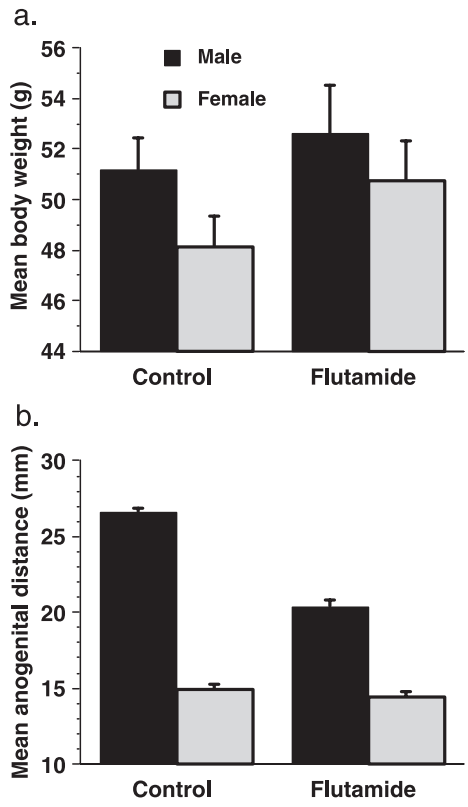

Fig. 1. (a) Mean (±S.E.) body weight (g) of male and female rats on postnatal Day 21 (litter means). (b) Mean (±S.E.) anogenital distance (mm) of males and females on Day 36 (litter means). In both groups the two treatment conditions were control injection ($n = 18$ litters) or flutamide injection ($n = 13$ litters) from Days 11–21 of gestation. See Results for descriptions of significant differences.

A 3 × 3 (Group × Session) mixed ANOVA was used to analyze body weight measured at the end of each play session. Significant effects for group, $F(2,93) = 19.19$, $P < .0001$, session, $F(2,186) = 8101.67$, $P < .0001$, and the group by session interaction, $F(4,186) = 52.35$, $P < .0001$, were found. Probing the group effect revealed that control females weighed less than control males, $t(62) = 51.24$, $P < .0001$, and flutamide, males, $t(62) = 23.06$, $P < .0001$. Control males did not differ significantly from flutamide males. Probing the session by group interaction indicated that control and flutamide males gained weight at a faster rate than control females.

3.1. Play behavior

The ontogeny of play behavior followed a previously reported pattern [13]. Peak levels of play occurred on Day 31 of age. This was also the only day on which characteristic sex differences in play behavior were apparent between control males and control females. Because sex differences between controls were not detected on Days 27 and 35, play data for these days were not analyzed further. On Day 31 a larger percentage of control males displayed three (pouncing, pinning, and boxing) of the five play patterns than control females (binomial tests, $P < .01$). The frequencies of occurrence of these three play components were summed to provide a single index of sexually dimorphic play behavior (see Fig. 2). The mean play frequencies reported in Fig. 2 were lower than expected. These low frequencies were due to a number of animals in each of the three groups that exhibited no play behaviors during the periods when they were the focal animal. Due to the number of nonresponding animals, nonparametric statistics were used to analyze play frequency. A Kruskal–Wallis H test on this index confirmed a significant group effect, $H = 8.88$, $P < .005$, and subsequent Mann–Whitney U tests indicated that flutamide-exposed males exhibited a significantly lower mean frequency of these combined play components than control males ($P < .01$), but did not differ from control females. As expected, control females also exhibited less frequent sexually dimorphic play than control males.

Approximately 50% of both control males and control females exhibited wrestling behavior, whereas significantly fewer (31%) of the flutamide-exposed males showed this behavior (binomial test, $P < .05$). The submissive behavior pattern, on-the-back, was exhibited by a greater percentage of the control females (34.4%) than either control males (12.5%, $P < .005$) or flutamide males (15.6%, $P < .05$).

Because of the number of nonresponders, nonparametric statistics were also used to analyze partner preference during

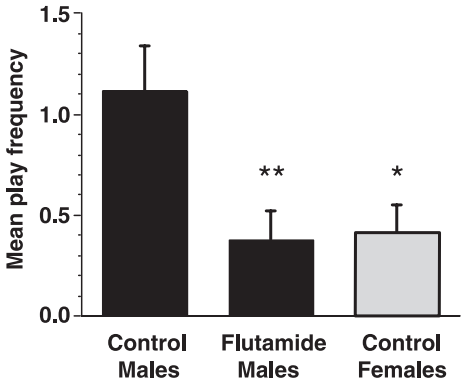

Fig. 2. Mean (±S.E.) play frequency (pounce, pin, and box) on postnatal Day 31. The three treatment conditions were control males ($n=32$), flutamide males ($n=32$), and control females ($n=32$). Asterisks indicate a value that differs significantly from that of control males (*$P<.05$, **$P<.01$).

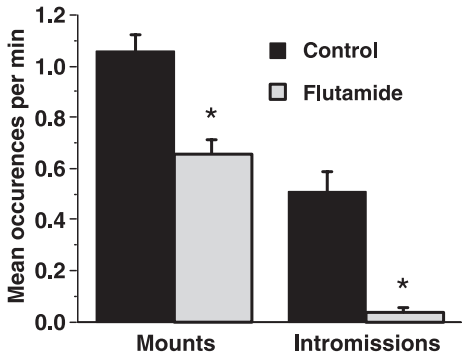

Fig. 3. Mean (±S.E.) mount and intromission frequencies of responding flutamide-exposed and control males. Frequencies were calculated per minute of "active time" (see Methods). Asterisks indicate values that differ significantly from controls ($P<.0005$).

play. Separate Kruskal–Wallis H tests were used to make within-group comparisons of preference for each type of partner (control male, flutamide male, and control female). No significant differences in the frequency of partner preference were found in any group.

3.2. Male sexual behavior

Prenatal flutamide exposure attenuated all three of the major components making up the male copulatory pattern. None of the flutamide males ejaculated on any of the three tests. In comparison, 84.4% of the control males ejaculated at least once.

Intromission behavior was also affected. Binomial tests indicate that a significantly higher percentage of control (93.8%) than flutamide males (28.1%) intromitted on at least one of the three tests ($P<.00001$). A one-way ANOVA was performed on the intromission frequencies of all responding (intromitting) animals. Control responders had significantly higher intromission frequencies (mean = 0.51 mounts/min) than flutamide responders (mean = 0.04 mounts/min), $F(1,37)=24.00$, $P<.0001$ (see Fig. 3).

There was no difference in the percentage (96.9% in each group) of control and flutamide males that mounted on at least one test. A one-way ANOVA was performed on the mount frequencies of all responding (mounting) animals. Controls had significantly higher mount frequencies than flutamide males, $F(1,60)=15.62$, $P<.0004$ (see Fig. 3).

3.3. Male morphological measures

The morphological measures taken on adult animals at 120 days of age are summarized in Table 1. The data from control and flutamide males were compared in separate one-way ANOVAs. The testes of control males weighed significantly more than the testes of flutamide males, $F(1,28)=15.25$, $P<.0008$. The testis data of two flutamide males were not included in the analysis due to bilateral infection and malformation. The epididymis weighed significantly more in control males than in flutamide males, $F(1,26)=20.63$, $P<.0003$. The epididymis data from four flutamide males were excluded from the analysis because the structure was bilaterally infected and/or malformed. The penis of control males was significantly longer than that of flutamide males, $F(1,30)=80.40$, $P<.0001$, and control males had a significantly longer AG distance in adulthood than flutamide males, $F(1,30)=186.76$, $P<.0001$. The body weight of adult control and flutamide males was not significantly different. Blind vaginal openings were noted only in flutamide males and averaged 8.0±0.92 mm in depth.

3.4. Plasma testosterone levels

There were no significant differences in plasma T concentrations between control and flutamide males (means 1.988±0.329 and 1.874±0.189 ng/ml, respectively).

Table 1
Mean (±S.E.) testis, epididymis, and body weight, and anogenital distance of control and flutamide-treated male and female rats at 120 days of age

	Control males ($n=16$)	Flutamide males ($n=16$)	Control females ($n=16$)	Flutamide females ($n=10$)
Left testis weight (g)	1.80 (0.03)	1.31* (0.14)		
Left epididymis weight (g)	0.68 (0.02)	0.47* (0.04)		
Penile length (mm)	7.83 (0.22)	5.34* (0.17)		
Anogenital distance (mm)	38.34 (0.73)	25.50* (0.60)	15.42 (0.42)	15.15 (0.45)
Body weight (g)	417.5 (6.01)	402.3 (9.96)	267.9 (3.25)	260.3 (4.59)

* Flutamide-treated males differ significantly from controls ($P<.001$).

4. Discussion

The present data are the first demonstration that rough-and-tumble play in juvenile male rats is incompletely masculinized following exposure to an androgen receptor antagonist during prenatal development. The exact mechanism by which this hypomasculinization of play occurred is unknown. However, the feminization of play patterns is not due to alterations in the gonadal steroid levels of the young animals because play behavior occurs before the onset of puberty and is unaffected by modifying the androgen levels of juvenile males either by castration or T injections [4,16]. The alterations in play were unrelated to body size. The weight of control and flutamide males did not differ at the ages when play behavior was being tested. The reductions in play fighting are most likely due to inadequate androgenization of CNS targets such as the amygdala that have been postulated to mediate sexually dimorphic play [17–19].

The feminization of rough-and-tumble play in males prenatally exposed to an androgen receptor blocker is similar to the reductions in play exhibited by prenatally stressed male rats [13,20]. The present data support the conclusion of Ward and Stehm [13] that although the organizational period for play behavior may end neonatally, it actually begins prenatally, possibly during Days 18–19 of gestation when a surge in plasma T normally occurs in male rats [21]. Prenatally stressed males lack the prenatal T surge [22–24], and exposure to flutamide also would have prevented androgen-sensitive CNS tissue from developing under the influence of an enhanced T milieu during this fetal stage. It is unlikely that the attenuation in play observed in males following prenatal stress is due to alterations in neonatal androgen levels because the neonatal T surge in stressed males is virtually identical to that of control males [25]. In the present study, it is unlikely that appreciable levels of flutamide or its active metabolite, 2-hydroxyflutamide, had any effect on androgen receptor occupation during the neonatal T surge of males prenatally exposed to flutamide, given the short terminal half-lives of these compounds [26]. Taken together with the prenatal stress studies, the current data provide support for the notion that prenatal androgen exposure contributes to the masculinization of juvenile play potentials.

Copulatory behavior in males prenatally exposed to flutamide was severely impaired. All three components of the male copulatory pattern (mounts, intromissions, and ejaculations) were incompletely masculinized. A decreased potential to execute intromissions and to ejaculate has been noted previously in males exposed to the antiandrogen flutamide [9,10]. However, in the present study, we also observed a sharp reduction in mount frequency. Detection of the deficit in mounting behavior is probably attributable to the amount of androgen in the males' circulation at the time they were being tested. In all previous work, the animals were castrated and male copulatory behavior was activated by an extended series of daily injections with the long-lasting synthetic androgen, TP. In the present study the animals were gonadally intact and were tested under the influence of T titers generated by their own testes. We have previously demonstrated that alterations in sexual behavior potentials due to prenatal hormonal reductions are more likely to be detected when rats are tested under physiological T levels than when tested under conditions that provide high, sustained occupation of androgen receptors [11,12].

Adult levels of plasma T at 400 days of age in the flutamide-exposed males were found to be no different from those in the control group. Thus, their deficient copulatory behaviors cannot be attributed to endogenous T levels too low to activate an otherwise normal behavioral potential. It is understood that endogenous T levels were measured when the males were older (400 days) than when behavior was being assessed (70+ days). Nevertheless, it is unlikely that group differences in T large enough to affect copulatory behavior in young adults would have been completely undetectable in more mature males. It seems more likely that depriving the developing CNS of normal androgen exposure during critical fetal stages may have selectively altered the threshold of activation of the neural mechanisms that mediate display of the several components of the male sexual pattern. The execution of intromissions and ejaculations are probably more heavily dependent on sensory feedback from a normal phallus than are mounting responses. As our data demonstrate, various dimorphic components of the male morphology, including the size of the penis, are incompletely masculinized in prenatal flutamide-exposed males. Mounting is a more generic response. Although it is an integral part of the full male copulatory pattern, mounting can often be activated in females by extensive priming with androgen, albeit lower response rates are usually exhibited [27]. Thus, treating flutamide males with high dosages of TP might mask an existing impairment in the androgen threshold needed to activate mounting. In the present study, such impairment became clearly apparent when the males were tested under lower, endogenous levels of plasma T.

The abnormal intromission and ejaculatory patterns exhibited by flutamide-exposed males may be due to inadequate masculinization of both the peripheral structures involved in the execution of male sexual behavior and to various components of the CNS that control these structures. To test the hypothesis that CNS changes are involved, the dorsolateral nucleus (DLN) and the spinal nucleus of the bulbocavernosus (SNB) of the lumbar spinal cord were examined in a sample of the flutamide-exposed males from the present study. A flutamide-induced reduction in the number of motor neurons was found in both CNS structures [28]. The DLN and SNB are sexually dimorphic spinal nuclei that innervate the muscle complex (ischiocavernosus, bulbocavernosus, and levator ani) that controls penile reflexes active during erection and ejaculation [29,30].

The mass of this muscle complex also was reduced in flutamide-treated males. These results, in conjunction with the present demonstration that penile length is reduced by prenatal flutamide, suggest that the phallus and musculature controlling it may have been inadequately developed to allow the display of normal intromissions or ejaculations [31,32]. Abnormalities in penile morphology including reductions in penile length have also been demonstrated recently in male hyenas exposed to antiandrogens during prenatal development [33]. As in flutamide-treated rats, when these male hyenas reach sexual maturity they exhibit marked decrements in their ability to intromit and ejaculate during mating attempts [34]. In rats, flutamide administered during the last week of gestation also reduces the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the male offspring [35]. The SDN-POA is larger in males than females [36], and its volume has been correlated with performance of the male copulatory pattern [37]. Furthermore, lesions of the SDN-POA suggest that it is involved in the regulation of sexual behavior in male rats [38].

Sexual differentiation of male copulatory behavior has been attributed to adequate exposure of the developing CNS to estradiol derived from the intracellular aromatization of T, during critical perinatal stages (see Ref. [32] for a review). Several lines of evidence suggest that blockage of androgen receptors with flutamide during prenatal development indirectly may have reduced intraneuronal estradiol, resulting in the observed deficits in copulatory potentials. Theoretically, flutamide could affect aromatization by altering the amount of substrate (T) and/or the expression of the aromatase enzyme. Flutamide exposure in the adult [39], prepubertal [40], and even early postnatal (Day 4) male rat [41], actually produces elevated levels of circulating LH and T, presumably because blocking androgen receptors reduces the negative feedback T normally exerts on the hypothalamic–pituitary–gonadal system. However, flutamide exposure does not increase the availability of the substrate for aromatization, i.e., testosterone on Day 18 [42] or Day 21 [41,42] of gestation. Flutamide does not alter plasma LH or T [41] or testicular T [42] during prenatal development. Although there is no change in substrate levels in flutamide-exposed fetuses, less estradiol still might be produced. Androgen occupation of T receptors stimulates expression of the aromatase enzyme in hypothalamic tissue during prenatal development in guinea pigs [43], mice [44] and rats [45]. Flutamide has been specifically shown to block T-induced increases in aromatase activity of hypothalamic neurons taken from fetal mice [44]. Thus, flutamide treatment probably reduces the availability of intraneuronal estradiol during the critical prenatal period of development.

Prenatal flutamide exposure clearly blocked masculinization of several androgen-dependent anatomical features. On Day 14, the flutamide-exposed males could not be distinguished from their female littermates or from control females on the basis of AG distance. In fact, males were indistinguishable from females on the basis of external appearance until 21 days of age. As in previous reports [9,10], the effect of prenatal flutamide on AG distance was not the result of a reduction in overall body mass. By 36 days of age, the flutamide-exposed males and females showed significant differences in AG distance, however, flutamide males were still hypomasculinized in comparison to control males. The reduction in AG distance persisted into adulthood.

Prenatal exposure to flutamide had other long-term residual effects on sexual morphology. Not only was the size of the penis reduced, but also a patent blind vagina was present in adult male rats prenatally exposed to flutamide. Further, despite an approximately 30% reduction in the weight of the testes (and epididymides), at 400 days flutamide males experienced no detectable reduction in adult T levels compared to controls. This finding is similar to that of a previous study that reported a 38% reduction in the adult weight of testes that had descended into the scrotal sac, but no reduction in testicular T [46]. In the latter study, twice as much prenatal flutamide (10 mg/day) was given as in the current study, and only about 50% of the testes descended normally. With the 5 mg/day dose used in the present study, no cryptorchidism was found in flutamide males.

In summary, while deficient development of the external genitalia may partially explain the reductions in intromittive and ejaculatory behavior of flutamide-exposed males, the incomplete masculinization of their play fighting and the reduction in nonintromittive mounting are more reasonably attributed to the effects the androgen antagonist exerted on sexual differentiation of the CNS during critical prenatal stages. At one time, it was assumed that because neonatally castrated males showed a complete failure of masculinization of copulatory behavior, sexual differentiation must occur exclusively in the neonatal period. It is now clear that prenatal steroid exposure is very important for the establishment of normal male behavioral potentials, probably by priming the CNS to be sensitive to later exposure to elevations in T that occur during neonatal ontogeny [21]. It appears that a similar scenario may hold for what is considered to be the "critical period" for the differentiation of play behavior. The current dogma that only the neonatal period is important is based on the dramatic influences that neonatal T manipulations have on the propensity to play. However, potential effects on play in females of exposure to T restricted to the prenatal period have not been investigated, and the current study is the first to restrict androgen receptor occupation in fetal males.

Acknowledgements

The authors thank Dr. Rudi Neri (of the Schering-Plough Corp., Bloomfield, NJ) for donating the flutamide, and Dr. G.D. Niswender for supplying the testosterone antiserum. Thanks also to Ingeborg L. Ward for her advisement and critical comments during the preparation of this manuscript.

Thanks also go to Rick Ward who wrote the computer programs used to summarize the data. This study was supported by Grant HD-04688 NICHHD to Ingeborg L. Ward.

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