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Effect of prenatal androgen receptor antagonist or aromatase inhibitor on sexual behavior, partner preference and neuronal Fos responses to estrous female odors in the rat accessory olfactory system

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Abstract

Many socially relevant odors are detected in rodent species by the vomeronasal organ and subsequently processed by the accessory olfactory system (AOS). We previously found that gonadectomized male and female rats treated in adulthood with testosterone propionate (TP) showed equivalent Fos responses in the AOS to odors derived from estrous females. Likewise, in contrast with numerous other mammalian species, gonadectomized female rats show surprisingly high levels of male-typical mounting behavior in response to adult TP. We tested the hypothesis that prenatal testosterone (T) exposure, acting via androgen receptors (ARs) or via estrogen receptors, masculinizes the AOS in rats of both sexes. Pregnant dams were treated with either the AR blocker, Flutamide, the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), or nothing (control) to assess the role of prenatal androgen and estradiol receptor activation, respectively, in this masculinization. Beginning at birth, male and female offspring were injected subcutaneously (sc) every other day with either ATD (pre- and neonatal ATD group) or oil vehicle (Flutamide and control groups) until postnatal Day 12. Subjects were gonadectomized as adults, hormonally treated and tested for different behaviors before having their AOS Fos responses to estrous female odors assessed. Prenatal treatment with Flutamide (but not ATD) significantly decreased anogenital distance and severely impaired intromissive and ejaculatory behaviors in males tested after TP replacement without disrupting mounting capacity in either sex. Pre- and neonatal treatment with ATD (but not Flutamide) enhanced lordosis responsiveness in males tested after sc injections of estradiol and progesterone, whereas these perinatal treatments had no effect on any aspect of masculine coital performance in either sex. After TP treatment, male and female control subjects preferred to approach a tethered stimulus female as opposed to a male, and prenatal Flutamide or perinatal ATD treatments did not modify this pattern of partner preference. Neuronal Fos responses to estrous odors were (as in previous studies) identical in the AOS of gonadectomized TP-treated control males and females. Prenatal Flutamide or perinatal ATD treatments failed to disrupt consistently this profile of Fos responses to estrous odors in the AOS of rats of either sex. These behavioral and neuroanatomical findings raise the possibility that the similar level of male-typical responsiveness to social odors that occurs in male and female rats after adult TP treatment results from nonsteroid-hormone-dependent, species-specific factors that act perinatally in the brains of rats of both sexes.

Keywords: Accessory olfactory system (AOS); 1,4,6-Androstatriene-3,17-dione (ATD); Flutamide; Partner preference; Estrous odors; Fos responses

1. Introduction

In many vertebrate species, olfactory communication is important for a variety of social functions, including the attraction of conspecifics prior to mating, delineation of territories and communication between mother and young. Numerous studies have shown that olfactory cues contribute to sexual partner preference in mice [1,2], rats [3–6], hamsters [7], voles [8,9] and ferrets [10]. When tested in a T-maze, adult male rats preferred to investigate the arm containing odors from an estrous as opposed to an anestrous female [3]. Likewise, when given free access to soiled bedding from sexually active males and estrous females,

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male rats preferred to investigate bedding soiled by estrous females. Female rats, on the other hand, showed a small preference for soiled bedding from sexually active males as opposed to estrous females [6].

Many, though not all, socially relevant olfactory cues are detected and processed in rodent species by a polysynaptic accessory olfactory system (AOS) [11,12]. Nonvolatile socially relevant odors are detected by receptors in the vomeronasal organ whose axons project to the glomeruli of the accessory olfactory bulb (AOB) [11]. Mitral cells in the AOB project to the anterior–dorsal medial amygdala (MeAD) and the posterior–dorsal medial amygdala (MePD). Neurons of the MeAD project via the ventral amygdalofugal pathway to the hypothalamic ventromedial nucleus and the lateral preoptic area (POA) whereas neurons in the MePD project via the stria terminalis to the bed nucleus of the stria terminalis (BNST) and the medial POA [13–15]. In rats, the AOS is sexually dimorphic with males having more neurons in several of the above mentioned brain regions, except the lateral POA (reviewed in Ref. [16]).

Immunocytochemical visualization of the nuclear protein product of the immediate-early gene, c-fos, has provided useful information about the neural pathways, which are activated in response to mating-associated behaviors (e.g. Ref. [17]). Numerous studies have also assessed the ability of chemosensory cues to augment neuronal Fos immunoreactivity in the AOS of mice [18–20], rats [21–23] and hamsters [24,25]. In hamsters, but not in rats, estrous female odors differentially activated neurons in the AOS of males and females. Thus, exposure to female hamster vaginal secretions (FHVS) induced Fos-IR in the MePD, BNST and the magnocellular medial preoptic nucleus in gonadectomized, testosterone-treated male hamsters. Ovariectomized female hamsters treated with testosterone showed significant increases in Fos-IR nuclei in the MePD, BNST, but not in the medial preoptic nucleus, after FHVS exposure [25]. By contrast, estrous female odors augmented Fos-IR equivalently throughout the AOS, including the MPOA, of both male and female rats that were gonadectomized in adulthood and treated with testosterone propionate (TP) [22,23]. These species differences may reflect differences in circulating testosterone during development. Female rat fetuses are exposed to considerable amounts of testosterone of placental origin [26,27], which may promote aspects of male-typical neural differentiation. We use the term 'male-typical' here to refer to neural mechanisms controlling behaviors such as mounting and pelvic thrusting, which are displayed by males of essentially all mammalian species. Females of many mammalian species show less of these male-typical coital behaviors than male conspecifics; however, female rats, when they are ovariectomized and treated with TP in adulthood, show levels of mounting that are surprisingly comparable to those shown by males [28–31]. By contrast, female hamsters are not exposed prenatally to male-like levels of testosterone [32], and in adulthood they have a limited capacity to display male-typical coital behaviors [25,33]. Likewise, the content of whole body androgen between embryonic (E) day 26 and birth (E 41) is significantly lower in female than in male ferrets, and when ovariectomized and treated in adulthood with TP female ferrets show very little male-typical mating behavior [34]. Taken together, these examples raise the possibility that the capacity of females of particular species (e.g. the rat) to show male-typical sexual behaviors in adulthood reflects their prenatal exposure to high levels of testosterone or to neural estrogenic metabolites of this androgen.

In the present study, we asked whether prenatal blockade of androgen receptor (AR) activation in either male or female rats using Flutamide would attenuate later neuronal Fos responses in the AOS to estrous odors. We recently found [21] that Fos responses in the AOS to odors derived from sexually active males are sexually differentiated and that neonatal administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) to male rats made their later Fos responses to male odors more female-like. Therefore, in the present study we also asked whether the pattern of AOS Fos responses to female odors (normally seen in both sexes when treated with TP) depends on perinatal aromatization of testosterone (T). To answer this second question we gave ATD prenatally and neonatally to groups of male and female rats. As adults, subjects were gonadectomized and received a battery of behavioral tests. We assessed female-typical lordotic responsiveness after treatment with estradiol benzoate plus progesterone. All subjects then received TP followed by tests of masculine coital behavior and of preference to approach and interact with a sexually receptive female versus a sexually active male in a three compartment apparatus. Finally, while they continued to receive TP, we assessed neuronal Fos responses to estrous odors in the AOS of all subjects.

2. Materials and methods

2.1. Animals and treatments

Male and female Sprague–Dawley rats obtained from the breeding colony at the Centro de Neurobiología (CNB, Universidad Nacional Autónoma de México [UNAM], Querétaro, México) were housed in single-sex groups of two to three animals. Food and water were available ad libitum. Rats were maintained in a room with a reversed light–dark cycle (12 h light/12 h dark; lights off at 08:00 h).

The same protocol as described by Brand et al. [35] was used with a few modifications. Female rats were timemated. At Day 12 of gestation, pregnant dams received under light ether anesthesia a subcutaneous (sc) implant of a silastic capsule (inner diameter 1.5 mm; outer diameter 2.1 mm; length: 5 cm) containing either ATD or nothing. An additional group of pregnant dams was injected twice daily (10 a.m. and 8 p.m.) with Flutamide (10 mg/kg,

dissolved in propylene glycol with 10% ethanol) from Day 12 of gestation until pups were born. Data from our laboratory indicate that no behavioral differences are present between the offspring of mothers implanted with an empty capsule and mothers injected with propylene glycol. Male and female offspring from ATD-treated mothers continued to receive ATD by injection (1 mg/rat; every other day with corn oil as the vehicle) until Day 12 after birth. Male and female offspring of Flutamide-treated and control mothers were injected every other day with vehicle until Day 12 after birth. Pups were weaned at 25 days of age and housed two to three of the same treatment and sex to a cage. At approximately 3 months of age, all subjects were gonadectomized under sterile conditions using ketamine (70 mg/kg) and xylazine (6 mg/kg).

Stimulus animals were sexually active males and estrous females. Male stimulus animals were gonadally intact and sexually experienced. Female stimulus animals were brought into behavioral estrus by injecting them with 25 μg of estradiol benzoate (EB) sc 48 h before testing and by an injection of 1 mg/kg progesterone (P) 4 h before mating tests or 6 h before the collection of bedding that was used to evaluate neuronal Fos responses.

2.2. Test procedure

Subjects treated with EB and P were tested twice for feminine sexual behavior with a sexually active male. Two weeks later, all subjects, males and females, received daily injections of TP. After 2 weeks of treatment, subjects were tested twice for masculine sexual behavior with an estrous female and twice for partner preference.

2.3. Behavioral testing

2.3.1. Female coital behavior

Subjects were tested for lordosis behavior using EB (25 μg, 52 h prior to testing) and P (1 mg/kg, 4 h before testing) as the activational steroid hormone. Each subject was tested twice for lordosis behavior on separate days; data from these 2 tests were combined. The lordosis responses and lordosis intensity (on a scale of 0–2; 0 = *no lordosis*, 1 = *partial lordosis* characterized by lateral tail deviation with moderate concave back flexion and neck extension, 2 = *full lordosis* characterized by lateral tail deviation with pronounced back flexion and neck extension) of experimental animals to the mounting of a stimulus male were scored. Tests lasted until the experimental animal had received 10 mounts (with or without intromission) by the stimulus male. Stimulus males that stopped mounting were replaced by other, active males. Usually subjects received 10 mounts or intromissions within 10 min. The lordosis quotient, LQ [(total number of lordosis responses/total number of mounts) × 100] and the mean lordosis intensity, LI (sum of points per test/number of mounts) were calculated.

2.3.2. Male coital behavior

Subjects were injected daily with TP (5 mg/kg) and beginning 14 days later were tested for masculine sexual behaviors with a behaviorally receptive female. The following sexual behaviors were scored: latency to the first mount and intromission, the number of mounts, intromissions and ejaculations, ejaculation latency and postejaculatory interval. In female subjects, which lack a penis, the terms intromission and ejaculation refer to behavioral patterns displayed that resemble those behaviors shown by males when they actually achieve penile intromission and/or ejaculate. Subjects were observed twice during 30-min tests that were separated by at least 2 days. Data from these two tests were combined.

2.3.3. Partner preference

While they continued to receive TP, subjects were tested twice for partner preference in a three-compartment box made of wood. The middle compartment (21 × 27 × 32 cm) was connected with the lateral compartments (36 × 27 × 32 cm) via a sliding door (10 × 10 cm). The lateral compartments contained in one side a sexually active male and in the other side an estrous female as stimulus animals. The stimulus animals were tethered to the rear of the compartment using a harness attached to a flexible rope. In this way, the tethered stimulus animals were able to display coital behaviors while being restricted to their respective compartments. During testing subjects were placed in the middle compartment, the sliding doors were removed after 1 min, and the time spent in each of the lateral compartments was recorded during a 10-min test. Data for the two tests were combined.

2.4. Neuronal Fos responses to estrous female odors

One day after the last behavioral test and injection of TP, male and female subjects that had received various perinatal treatments were randomly selected to be placed alone in a plastic cage (25 × 50 × 15 cm) that contained either clean or soiled estrous female bedding. The estrous bedding was collected from groups of five EB-treated stimulus females 6 h after they had received an injection of progesterone. Soiled bedding was used within 4 h after collection. These stimulus females had not been mated or used in behavioral tests for at least a week. Fresh sawdust was used as control bedding. After exposure to bedding for 90 min, subjects were anesthetized with sodium pentobarbital (100 mg/kg). The heart was exposed and the subject was perfused through the ascending aorta with 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 1 min followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 6 min. The brains were removed and postfixed in 4% paraformaldehyde for 2 h before they were placed in 30% sucrose/PBS solution for cryoprotection. Brains were frozen on dry ice and cut in the coronal plane at 35 μm using a sledge microtome. Free-floating sections were pretreated with 1% hydrogen peroxide/PBS solution for

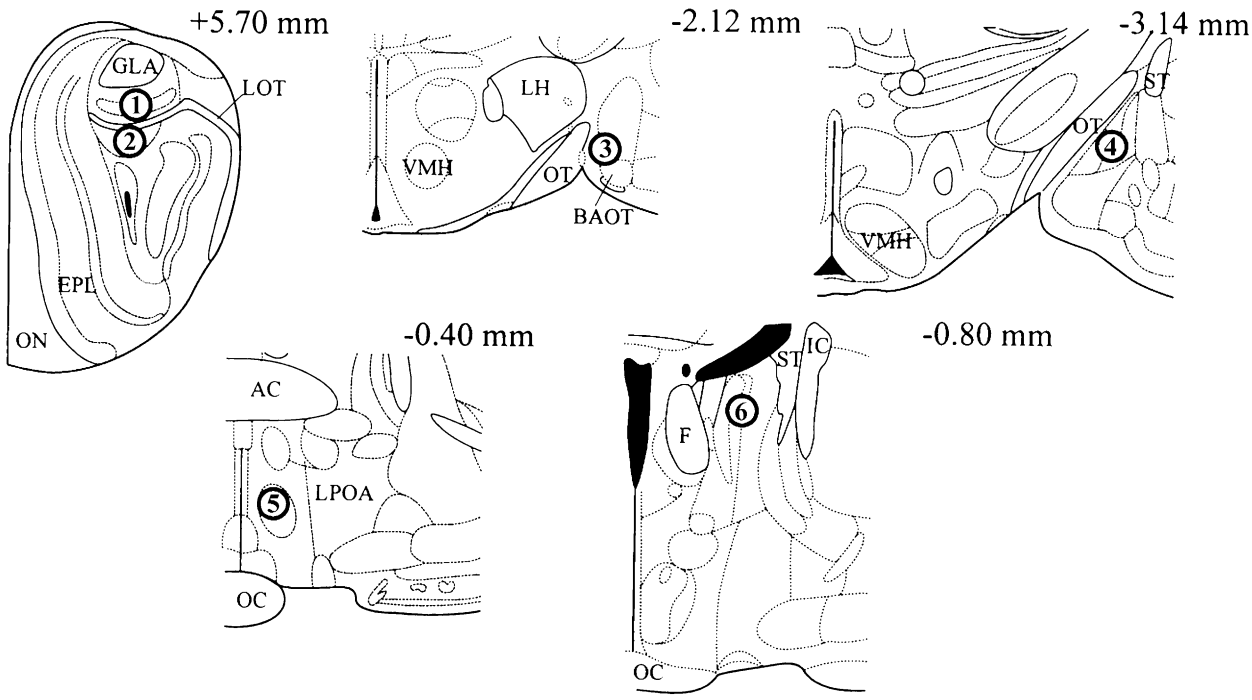

Fig. 1. Schematic diagrams showing the location of brain regions (dark circles containing numbers) in which Fos-IR cells were mapped and counted. 1, Mitral cell layer of the accessory olfactory bulb (AOB); 2, granular cell layer of the AOB; 3, anterior–dorsal medial amygdala (MeAD); 4, posterior–dorsal medial amygdala (MePD); 5, medial preoptic area (MPOA); 6, bed nucleus of the stria terminalis (BNST). GLA, glomerular layer of the AOB; LOT, lateral olfactory tract; EPI, external plexiform layer of the MOB; ON, olfactory nerve layer; AC, anterior commissure; LPOA, lateral preoptic area; OC, optic chiasm; IC, internal capsule; ST, stria terminalis; F, fornix; VMH, ventromedial nucleus of the hypothalamus; OT, optic tract; LH, lateral hypothalamus; BAOT, bed nucleus of the accessory olfactory tract. The distance (in millimeters) of each coronal brain section from bregma is given. Adapted from Ref. [23].

30 min. They were then rinsed with PBS and incubated overnight at room temperature in primary Fos antiserum diluted 1:5000 in PBS/0.52% Triton X-100 (DCH-1, a polyclonal antiserum raised in rabbit against the N-terminal sequence of rat Fos amino acids 2–17 (provided by Drs. Gerard Evans and David Hancock)). Brain sections were subsequently washed four times in PBS and incubated for 2 h at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlington, CA; 1:200 in 0.1 M PBS/0.52% Triton X-100). Sections were then rinsed four times in PBS and incubated in ABC solution (Elite kit; Vector Laboratories) for 1.5 h at room temperature. Sections were rinsed four times in PBS and reacted with nickel chloride–3,3′-diaminobenzidine and 0.0003% hydrogen peroxide for 5–10 min (DAB kit, Vector Laboratories). The sections were rinsed three times, mounted onto gelatin-coated slides and cover slipped using Permount.

To quantify the numbers of Fos-immunoreactive (IR) nuclei, all slides were coded so that the investigator had no knowledge of sex and treatment of the subjects. For each subject, one hemisphere from one brain section was chosen for analysis. Brain sections were selected at the level of the AOB (mitral and granule cell layers), the MeAD, MePD, BNST and medial POA (Fig. 1). Fos-IR nuclei were counted in an area of 0.22 mm^2 using an image-analysis system (Image-pro, Media Cybernetics, Silver Springs, MD) with a Hitachi digital color camera attached to an Olympus BX60 microscope.

Anogenital distances were measured when animals were anesthetized before brain perfusion.

Table 1
Effect of Flutamide or ATD treatment on anogenital distance in female and male rats

		Anogenital distance (cm)		Body weight (g)		(AG/BW) × 100	
Group	*n*	Females	Males	Females	Males	Females	Males
Control	11	1.77 ± 0.07	3.77 ± 0.16†	282 ± 13	343 ± 18†	0.63 ± 0.02	1.11 ± 0.03†
Flutamide	11	1.72 ± 0.09	2.34 ± 0.13**,†	304 ± 25	322 ± 24	0.57 ± 0.01	0.74 ± 0.03**,†
ATD	11	1.78 ± 0.08	3.86 ± 0.18†	284 ± 18	356 ± 24†	0.64 ± 0.02	1.10 ± 0.03†

Data are expressed as mean ± SE.

** Significantly different from control and ATD males; $P < .01$.

† Significantly different from females within the same treatment group; $P < .05$.

Table 2
Effect of Flutamide or ATD treatment on masculine and feminine coital behaviors in gonadectomized, TP-treated female and male rats

		Masculine sex behavior			Feminine sex behavior	
	n	Mount	Intromission	Ejaculation	LQ	LI
Females						
Control	11	15 ± 4	2 ± 1*	0 ± 0*	79 ± 8†	1.5 ± 0.2†
Flutamide	11	16 ± 3	1 ± 1*	0 ± 0*	88 ± 4†	1.7 ± 0.1†
ATD	11	15 ± 3	2 ± 1*	0 ± 0*	76 ± 7†	1.4 ± 0.2†
Males						
Control	11	28 ± 6	8 ± 2	1.2 ± 0.3	15 ± 9	0.3 ± 0.2
Flutamide	11	21 ± 4	1 ± 0.5*	0.1 ± 0.1*	11 ± 4	0.2 ± 0.1
ATD	11	33 ± 5	12 ± 2	1 ± 0.2	67 ± 9†	1.2 ± 0.2†

Data are expressed as mean number ± SE.
LQ, lordosis quotient; LI, lordosis intensity.
* Significantly different from control and ATD-treated males; $P < .05$.
† Significantly different from control and Flutamide-treated males; $P < .05$.

2.5. Statistics

Data from anogenital distance, feminine and masculine sexual behavior were evaluated by a 3 (group) × 2 (sex) ANOVA. A 3 (group) × 3 (compartment)-factor ANOVA for each sex was used to evaluate partner preference. Fos data were evaluated using a 3 (group) × 2 (bedding) factor ANOVA for each sex. All ANOVAs were followed by post hoc comparisons using the Fisher procedure.

3. Results

3.1. Anogenital distance

Prenatal treatment with Flutamide significantly decreased anogenital distance in male rats compared to control and pre- and neonatally ATD-treated subjects (Table 1). There were significant differences in anogenital distance, body weight and in the anogenital distance/body weight ratio between males and females within the same group. The only exception was that there were no significant differences in body weight between males and females treated with Flutamide.

The ANOVAs revealed a significant effect in anogenital distance [group $F(2,60) = 25.11$, $P < .0001$; sex $F(1,60) = 236.62$, $P < .0001$; Group × Sex $F(2,60) = 21.37$, $P < .0001$], body weight [group $F(2,60) = 0.09$, $P =$ ns; sex $F(1,60) = 10.53$, $P = .001$; Group × Sex $F(2,60) = 1.08$, $P =$ ns] and in the anogenital distance/body weight ratio [group $F(2,60) = 50.55$, $P < .0001$; sex $F(1,60) = 335.63$, $P < .0001$; Group × Sex $F(2,60) = 23.68$, $P < .0001$].

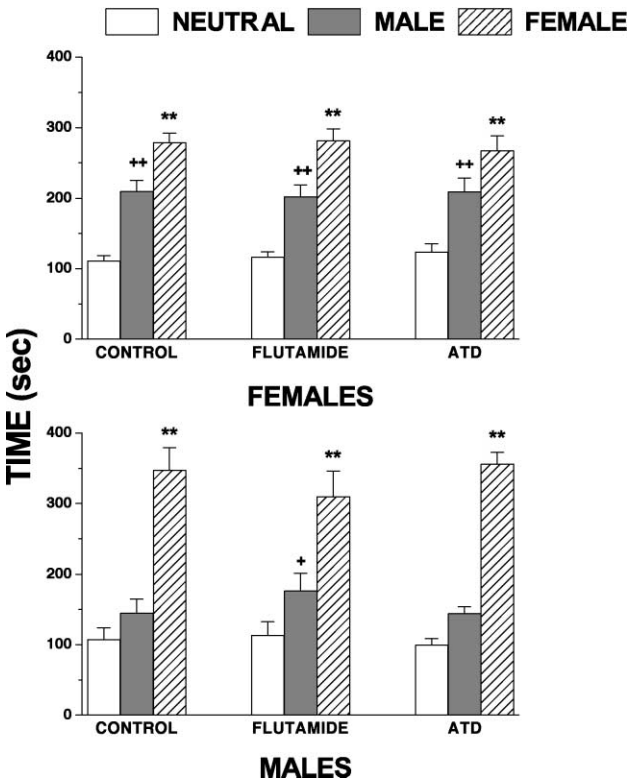

Fig. 2. Time spent in the compartment containing the estrous female versus the compartment containing the sexually active male versus the neutral compartment by prenatally Flutamide or perinatally ATD-treated males and females. Animals were gonadectomized in adulthood and received daily injections with TP. Data are expressed as mean ± S.E.M. ** Significantly different from time spent in the neutral compartment and from the time spent in the compartment containing the sexually active stimulus male, $P < .01$. + Significantly different from time spent in the neutral compartment, $P < .05$; ++ $P < .01$.

3.2. Feminine coital behavior

Pre- and neonatal treatment with ATD attenuated the defeminization of lordotic responsiveness to ovarian hormones that otherwise occurred in control males. Thus, perinatally ATD-treated males had lordosis quotients that were similar to those seen in all groups of females (Table 2) [group $F(2,60) = 6.39$, $P = .0032$; sex $F(1,60) = 65.59$, $P < .0001$; Group × Sex $F(2,60) = 11.07$, $P < .0001$]. Furthermore, lordosis intensities were significantly greater in perinatally ATD-treated males compared to control and prenatally Flutamide-treated males [group $F(2,60) = 3.99$, $P = .023$; sex $F(1,60) = 58.19$, $P < .0001$; Group × Sex $F(2,60) = 7.89$, $P = .0009$]. Lordosis quotients and intensities were similar among groups of females (Table 2).

3.3. Masculine sexual behavior

Prenatal treatment with Flutamide severely impaired later intromissive and ejaculatory behaviors in males, but not in females (Table 2). By contrast, mounting behavior was not affected by prenatal Flutamide treatment in either sex. Intromission and ejaculation frequencies were significantly higher in control and perinatally ATD-treated males compared to prenatally Flutamide-treated males and all female groups [intromissions: group $F(2,37) = 4.66$, $P = .015$; sex $F(1,37) = 16.27$, $P = .0003$; Group × Sex $F(2,37) = 5.73$, $P = .0068$; ejaculations: group $F(2,37) = 3.21$, $P = .051$; sex $F(1,37) = 16.00$, $P = .0003$; Group × Sex $F(2,37) = 3.21$, $P = .051$]. There was only an effect of sex on mounting behavior, with males displaying more mounts than females [group $F(2,60) = 0.33$, $P = .702$; sex $F(1,60) = 5.19$, $P = .026$; Group × Sex $F(2,60) = 0.47$, $P = .62$].

3.4. Partner preference

When given TP in adulthood, all subjects showed a clear preference for an estrous female as opposed to a sexually active male (Fig. 2). There was a significant difference between time spent in the compartment containing the female versus time spent in the compartment containing the sexually active male or the neutral compartment [females: group $F(2,90) = 0.00$, $P = 1$; compartment $F(2,90) = 84.3$, $P = .0001$; Group × Compartment $F(4,90) = 0.24$, $P = .91$; males: group $F(2,90) = 0.00$, $P = 1$; compartment $F(2,90) = 91.15$, $P = .0001$; Group × Compartment $F(4,90) = 1.01$, $P = .40$]. Post hoc comparisons showed that female subjects and prenatally Flutamide-treated males spent significantly more time in the compartment containing the sexually active male than in the neutral compartment.

3.5. Neuronal Fos immunoreactivity to estrous female bedding

After exposure to soiled estrous female bedding, significant increments in the number of Fos-IR neurons were seen in various segments of the AOS of males and females

Table 3
Mean (±SE) number of Fos-IR cells in different brain regions included in the AOS of female and male subjects treated perinatally with either Flutamide or ATD

	AOB		Medial amygdala			
	Mitral	Granular	Anterior	Posterior	BNST	POA
Females						
Control						
Clean	12±3 (6)	1±0 (6)	9±6 (6)	5±2 (6)	6±2 (6)	6±1 (6)
Estrous	18±3 (5)	42±8 (5)**	6±2 (5)	14±4 (5)*	8±2 (5)	18±3 (4)*
Flutamide						
Clean	12±8 (5)	7±3 (5)	4±1 (5)	3±2 (5)	2±1 (5)	9±3 (5)
Estrous	16±5 (6)	13±3 (6)	7±3 (6)	8±3 (5)	2±1 (5)	20±6 (6)*
ATD						
Clean	14±11 (5)	3±1 (5)	4±2 (5)	3±1 (4)	2±1 (5)	2±1 (5)
Estrous	31±13 (6)	44±13 (6)**	6±4 (5)	13±3 (5)*	2±1 (6)	33±8 (6)**
Males						
Control						
Clean	18±6 (6)	3±2 (6)	5±1 (6)	1±1 (6)	5±2 (5)	6±1 (6)
Estrous	20±3 (5)	30±5 (5)**	8±3 (5)	13±3 (5)**	9±2 (5)	20±4 (4)**
Flutamide						
Clean	7±2 (6)	2±1 (6)	3±1 (6)	1±0 (6)	0±0 (6)	3±1 (6)
Estrous	17±4 (5)	21±8 (5)**	9±4 (5)	8±2 (5)	4±3 (5)	10±3 (5)*
ATD						
Clean	21±8 (5)	14±2 (5)	6±4 (5)	6±2 (5)	4±1 (5)	8±2 (5)
Estrous	22±5 (6)	39±6 (6)**	4±2 (6)	16±2 (6)**	1±1 (6)	16±2 (6)*

The number of animals is given in parentheses.
* Significantly different from clean bedding ($P < .05$).
** ($P < .01$).

of all treatment groups except for Flutamide-treated females (Table 3). Even in the latter group of females the number of Fos-IR cells in most brain regions tended to be higher in animals killed after exposure to estrous as opposed to clean bedding. Only in one region, the POA, was the number of Fos-IR cells significantly greater ($P = 0.052$) by a post-hoc test in Flutamide-treated females exposed to estrous as opposed to clean bedding. Table 3 shows that the mean numbers of Fos-IR cells in several brain regions after exposure to estrous odors did not differ between Fluta-

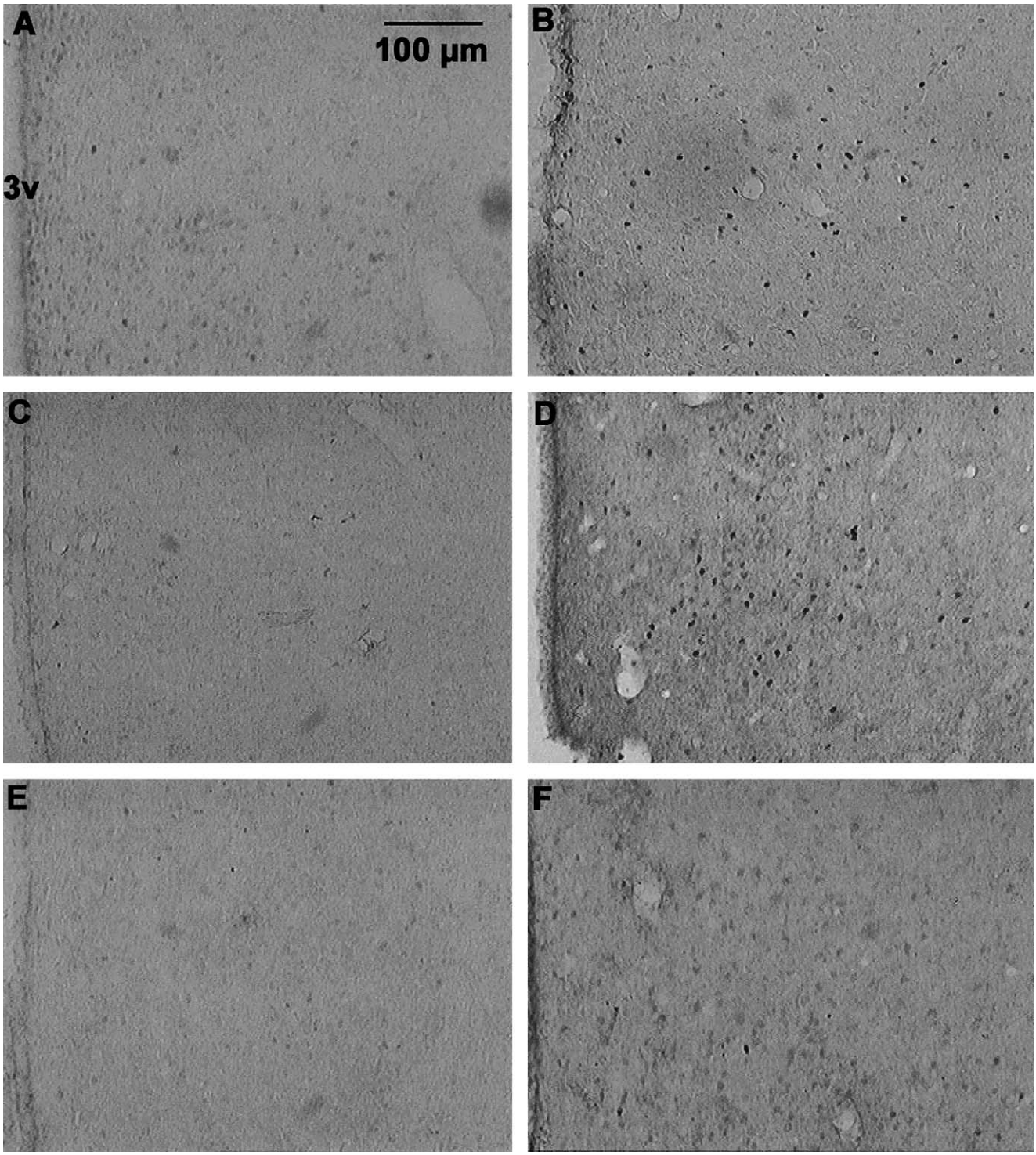

Fig. 3. Representative photomicrographs showing neuronal Fos-IR in the MPOA of female subjects prenatally treated with oil (A and B), ATD (C and D) or Flutamide (E and F). Subjects were gonadectomized as adults and received daily injections with TP before they were exposed to clean (A, C and E) or soiled estrous bedding (B, D and F). 3v, third ventricle.

mide-treated and control females. These results suggest that in females treated prenatally with Flutamide, as in control as well as ATD-treated female subjects (and in all groups of males), exposure to estrous odors stimulated neuronal Fos-IR in the AOS.

Statistical analysis showed a significant effect of bedding in the granular layer of the AOB [females: $F(1,28) = 42.10$, $P < .0001$; males: $F(1,28) = 34.69$, $P < .0001$]; the MePD [females: $F(1,26) = 12.39$, $P = .0017$; males: $F(1,28) = 26.53$, $P < .0001$] and the POA [females: $F(1,27) = 17.93$, $P = .0003$; males: $F(1,27) = 26.00$, $P < .0001$]. Examples of neuronal Fos responses to estrous female bedding are shown in Fig. 3.

4. Discussion

Our finding that Flutamide reduced anogenital distance and anogenital distance/body weight ratios in males when compared to control and ATD-treated males resembles those previously reported by Clemens et al. [36]. In that study Flutamide blocked the masculinizing action (longer anogenital distance and increased mounting behavior potential) in females that developed in utero between two males. Likewise, in Clemens et al. [36] and in the present study, males that were treated prenatally with Flutamide and later gonadectomized and treated with TP showed a drastic reduction in intromissive behavior. Similar results have been described after prenatal treatment with the anti-androgen, cyproterone acetate [37]. The reduction in intromissive behavior appears to be associated to an abnormal development of male penis in animals in which androgens are blocked perinatally [38].

In addition, the effects of perinatal ATD were also consistent with those previously described [21,35]. That is, perinatal treatment with ATD greatly facilitated feminine sexual behavior in male subjects that were later gonadectomized and treated with EB and P. Perinatally ATD-treated males had similar lordosis quotients and displayed lordosis with the same intensity as all groups of females. Considered together, these results clearly indicate that our prenatal treatments with Flutamide and ATD successfully reached the gestating fetuses.

Despite the successful prenatal blockade of AR by Flutamide or the perinatal interruption of estradiol biosynthesis by ATD, there was a surprising persistence of responsiveness to female bedding in subjects of both sexes that were treated in adulthood with TP. Neuronal Fos-IR after exposure to soiled bedding from estrous females was similar in the AOS of control males and females, confirming previous observations [22,23]. In those earlier studies, gonadectomized TP-treated males and females showed a preference for estrous as opposed to anestrous or clean bedding and similar increments in neuronal Fos-IR throughout the AOS when exposed to estrous odors [22,23]. The latter study [23] also showed that testosterone is required in order for gonadectomized male and female rats to show significant increments in neuronal Fos-IR throughout the AOS after exposure to soiled estrous bedding. Gonadectomized animals treated with oil showed no preference for estrous bedding and subsequently no increments in Fos-IR in the AOS [23]. Likewise, in the male hamster [39] mating behavior is activated by integration in the AOS of the actions of testosterone and olfactory signals from FHVS. In the present study, prenatal Flutamide treatment had no consistent disruptive effects on the responsiveness of the AOS to estrous odors in either male or female rats. This persistence of responsiveness to female bedding in subjects in which AR activation was attenuated during fetal life argues against our original hypothesis that both male and female rats' brains are masculinized during fetal life in response to testosterone derived from the placenta (both sexes) as well as the testes (males only).

Fos responses were similar in the AOS of perinatally ATD-treated males and females and in control animals of both sexes, suggesting that there was no effect of perinatal estrogen deprivation on subjects' later neural responsiveness to odors from estrous females. Similar results were described for neonatally ATD-treated male rats that were castrated in adulthood and given estradiol [21]. Taken together, these results further suggest that the functional activity of the AOS to odors from females is not affected by perinatal actions of estradiol in the developing brain. This outcome differs from the case of AOS responses to male odors for which a clear-cut sexual dimorphism was revealed [21]. In that study, exposure to male odors augmented neuronal Fos-IR in the BNST and the medial POA of female subjects, but not in males that were gonadectomized and given EB in adulthood. By contrast, males treated neonatally with ATD later showed female-like Fos-IR responses in both of these brain regions [21]. The lack of a significant disruption by prenatal Flutamide or by perinatal ATD treatments on the ability of estrous female odors to stimulate Fos in the AOS and in subjects' preference to approach an estrous female raises the possibility that these male-typical characteristics occur in both male and female rats as a result of some nonsteroid-dependent, species-specific process that acts during development in both sexes.

There is a body of evidence suggesting that differentiation of the male-typical profile of partner preference depends on the neonatal action of estradiol in the male brain [21,35,37,40–42]. Thus, perinatal treatment with ATD dramatically decreased the preference of adult male rats for an estrous female over a sexually active male. However, in the present study, partner preference was not altered by pre- and neonatal ATD treatment in either sex. All male and female groups preferred an estrous female as opposed to a sexually active male (albeit male subjects did show a stronger preference for the estrous female than did female subjects). The present study differed in two ways from previous studies [21,35,42]. First, male and female subjects used in the present study were gonadectomized and treated with high doses of TP prior to partner preference testing

whereas subjects were previously tested for partner preference either when gonadally intact [35,42] or when gonadectomized and primed with estradiol [42]. It has previously been shown that ovariectomized female rats treated with high doses of testosterone switched their preference to an estrous female when given free access to an estrous female versus a sexually active male [40]. Indeed a single injection of 500 μg TP to ovariectomized female rats stimulated a preference for an estrous female over a stimulus male [43]. Second, in the present study all behavioral testing was conducted during the second half of the dark phase of the light–dark cycle. It was previously found that neonatal ATD treatment to male rats induced nocturnal fluctuations in coital performance and sexual partner preference [44,45]. When tested in the first half of the dark phase, neonatally ATD-treated male rats showed feminine sexual behaviors while showing a preference for a sexually active male over an estrous female. By contrast, when tested late in the dark phase, ATD-treated males readily showed masculine sexual behaviors, including ejaculation, and preferred to interact with an estrous female as opposed to a sexually active male — thereby resembling control males.

In a previous study [37], no facilitation of lordosis behavior was observed in females that had been treated prenatally with the antiandrogen, cyproterone acetate. Likewise, in the present experiment no facilitation of feminine sexual behavior was observed in Flutamide-treated subjects. These observations are at variance with previous studies in which higher lordosis quotients were observed in prenatally Flutamide-treated male and female rats when they were given ovarian hormones and tested in adulthood [46]. One possible explanation for these differences is that in the present study subjects were injected only once with EB 52 h prior to testing whereas Gladue and Clemens [46] gave EB 72, 48 and 24 h before testing. Another important difference is that Gladue and Clemens [46] injected Flutamide once a day to pregnant mothers. In the present experiment, we injected Flutamide twice a day because of the short serum half-life of the compound [47].

Our results corroborate those of two earlier studies by Brand and Slob [40,41] in which prenatal administration of Flutamide failed to disrupt the later capacity of female rats, treated with TP in adulthood, to show a male-typical preference to approach estrous females in choice tests and to display high levels of mounting behavior in tests with estrous females. In the present study prenatal Flutamide treatment of male as well as female rats also failed to disrupt their later preference to approach an estrous female or to display mounting behavior. These results argue against any essential role for neural AR activation during prenatal life in the differentiation of the neural systems that control motivational aspects of masculine sexual behavior in either sex. It would be interesting to assess these functions (along with olfactory responsiveness to estrous odors) in male and female rats treated with a regimen of antiandrogenic drug treatment that completely blocks the masculinization of the male's external genitalia. This result will assure that the treatment completely blocked ARs and would provide a stronger test of the contribution of AR activation to the prenatal development of neural systems controlling these male-typical behavioral functions. Finally, it is possible that drug-induced reductions in neural AR activation are compensated for by the persisting activation of estradiol receptors in Flutamide-treated rats. It would be interesting to test this possibility by assessing the effects of combined prenatal treatment with Flutamide and ATD on later male-typical behaviors and neuronal Fos responses to estrous odors.

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