

## Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats

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### Abstract

The medial preoptic area (MPOA) is an important integrative site for male sexual behavior. Dopamine (DA) is released in the MPOA of male rats shortly before and during copulation. In a previous study, we identified 17 $\beta$ -estradiol (E<sub>2</sub>) as the metabolite of testosterone (T) that maintains MPOA basal extracellular DA levels. However, the presence of dihydrotestosterone (DHT), an androgenic metabolite of T, is required for the female-induced increase in MPOA DA observed during copulation. Recently, we reported that assays of MPOA tissue DA content showed that castrates actually had more stored DA than did gonadally intact males. Therefore, the reduction in extracellular levels in castrates was not due to decreased availability of DA; most likely it was due to decreased release. Furthermore, T upregulates neuronal nitric oxide synthase (nNOS) in the MPOA. NO has been implicated in the regulation of DA release in the MPOA. It is not known, however, which metabolite(s) of T regulate(s) tissue stores of DA and/or nNOS in the MPOA of male rats.

The present experiments were designed to test the following: (1) whether E<sub>2</sub>, DHT, or the combination of the two influences MPOA DA tissue levels, an indication of stored DA, in male rat castrates; and (2) whether E<sub>2</sub>, DHT, or the combination of the two influences NOS-ir in the MPOA of castrated male rats.

The results indicate that E<sub>2</sub> up-regulates nNOS-ir in the MPOA and maintains tissue content of DA at levels similar to those in T-treated rats. DHT did not influence nNOS-ir, while attenuating the effect of castration on tissue DA content.

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Dopamine (DA) in the medial preoptic area (MPOA) facilitates male rat sexual behavior (reviewed in Hull et al., 2002; Melis and Argiolas, 1995). MPOA DA is released in the presence of a receptive female and during copulation (Hull et al., 1995). The recent presence of testosterone (T) is necessary for this precopulatory increase in DA release; postcastration loss of copulatory ability mirrored the loss of the MPOA DA response to an estrous female (Hull et al., 1995), and restoration of copulatory ability with systemic T was concurrent with the reemergence of this response

(Putnam et al., 2001; Sato et al., 1998b). The two major metabolites of T, 17 $\beta$ -estradiol (E<sub>2</sub>) and dihydrotestosterone (DHT), differentially affected extracellular DA (Putnam et al., 2003). E<sub>2</sub> + DHT- and T-treated animals had high basal extracellular DA and showed the precopulatory MPOA DA response; most copulated to ejaculation. E<sub>2</sub>-treated castrates had normal basal DA levels but failed to show an increase in DA release in the presence of a receptive female; they intromitted but failed to ejaculate. DHT- and oil-treated castrates had low basal levels of extracellular DA that did not increase during copulation testing; a few of these animals mounted, but only one DHT-treated animal intromitted. Thus, normal basal extracellular DA levels in E<sub>2</sub>-treated males were associated with suboptimal copulation, but a female-stimulated increase in DA, apparently dependent on DHT, appeared to be necessary for the full expression of copulatory behaviors.

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Lower extracellular DA could result from either decreased synthesis or decreased release. Assays of tissue DA content in MPOA punches, representing mostly vesicular storage, showed that castrates actually had more stored DA than did gonadally intact males and showed greater DA release in response to amphetamine (Du et al., 1998). Therefore, the reduction in extracellular DA was not due to decreased availability, but most likely to decreased release. Experiment 1 tested which metabolite(s) of testosterone influence(s) tissue levels of DA in the MPOA.

Nitric oxide (NO), a highly reactive endogenous gas with a short half-life, is produced when L-arginine is converted to citrulline by the enzyme nitric oxide synthase (NOS). NO plays an important role in a variety of biological functions, including neurotransmitter release (Prast and Philippu, 2001; West et al., 2002). L-arginine, the precursor of NO, increased basal extracellular MPOA DA (Lorrain and Hull, 1993), an effect not seen with the inactive isomer, D-arginine. This increase was blocked by co-administration of the NO synthesis inhibitor *N*-monomethyl-L-arginine (L-NMMA). Furthermore, NO increased during copulation (Sato et al., 1998a) and mediated the increase in DA release normally seen during copulation (Lorrain et al., 1996). Such increase in DA was prevented by reverse dialysis of L-nitroarginine methyl ester (L-NAME), another NO synthesis inhibitor, but not the inactive isomer, D-NAME. Furthermore, we have recently shown that L-NAME interfered with copulation in both sexually naïve and experienced rats, when administered into the MPOA (Lagoda et al., 2004). We have also shown that the glutamate-induced DA release in the MPOA is mediated by NO (Dominguez et al., 2004). Therefore, NO may not only maintain basal DA levels but also promote the characteristic increase in MPOA extracellular DA in response to an estrous female and during copulation.

The predominant isoform of NOS in the brain is neuronal NOS (nNOS; Stuehr, 1999). nNOS in the MPOA is hormonally regulated. Fewer nNOS-immunoreactive (ir) neurons were found in the MPOA of male rats castrated 1 or 2 months earlier and injected daily with oil, compared to either castrates treated with T or gonadally intact males (Du and Hull, 1999). Such down-regulation of nNOS-ir was also observed in castrated male hamsters (Hadeishi and Wood, 1996) and mice (Scordalakes et al., 2002). Testosterone's effects on nNOS may be due to one of its metabolites, E<sub>2</sub> or DHT, rather than to T itself. The aim of Experiment 2 was to determine which metabolite(s) of T maintain(s) the number of nNOS-ir neurons in the MPOA, thus facilitating DA release and copulation.

## Materials and methods

### Animals

Adult male Long–Evans rats (250–300 g) were purchased from Harlan (Indianapolis, IN). Each rat was

individually housed in a clear plastic cage in a temperature- and humidity-controlled environment, on a 14:10 reverse light cycle with lights off at 11:00 h. Food and water were available ad libitum. Animals were weighed daily to monitor health status and to accustom them to handling. All animals were screened for copulatory ability 1 week after arrival.

Female rats of the same strain were ovariectomized under ketamine hydrochloride (47 mg/kg) and xylazine hydrochloride (4 mg/kg) anesthesia. They were brought into behavioral estrus with subcutaneous injections of 17 $\beta$ -estradiol benzoate (EB, 20  $\mu$ g, Sigma-Aldrich, St. Louis, MO) for 48 h and progesterone (P, 500  $\mu$ g, Sigma-Aldrich, St. Louis, MO) for 4 h prior to testing. Behavioral receptivity of each female was verified before copulation testing by permitting three intromissions by a stud male in his home cage.

### Copulation screening of test males

For copulation screening of the test males, a proven estrous female was placed in the home cage of each subject for 30 min, during which time mounts, intromissions, and ejaculations were recorded. Testing was considered complete when three ejaculations were achieved. If after four sessions an animal had not ejaculated three times, it was not used for this study.

### Surgery

After copulation testing, all sexually active male rats were anesthetized with ketamine hydrochloride (50 mg/kg im) and xylazine hydrochloride (4 mg/kg im) and castrated. A longitudinal midscrotal incision was made, and the testes were tied off and removed with a cut distal to the ligature. Bacitracin ointment was applied to the incision site to prevent infection. Each subject also received a subcutaneous injection of the antibiotic gentamicin (0.03 mg/kg) immediately after surgery and was weighed and checked daily for signs of infection. None of the animals experienced serious weight loss (>20 g) or showed any other signs of illness. All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Institutional Animal Care and Use Committee.

### Hormone replacement

Animals were randomly assigned to one of four hormone treatment groups or to a control (oil) group. Each received daily subcutaneous injections of either 20  $\mu$ g EB, 500  $\mu$ g dihydrotestosterone benzoate (DHTB, Sigma-Aldrich, St. Louis, MO), a combination of 20  $\mu$ g EB and 500  $\mu$ g DHTB, or 500  $\mu$ g testosterone propionate (TP, Sigma-Aldrich, St. Louis, MO) in 0.1 ml olive oil, or with 0.1 ml oil for the control group, for a period of 3

weeks beginning the day after castration. The hormone doses were chosen to be consistent with our previous studies.

### Experimental design

In both Experiments 1 and 2, animals were divided for statistical analysis based on the presence or absence of  $E_2$  ( $E_2+$  and  $E_2-$ ) and DHT (DHT+ and DHT-). The purpose of this study was to examine the relative contributions of estrogenic and androgenic metabolites of T on behavioral, anatomical, and biochemical measures. Therefore, we analyzed all animals except the TP-treated animals based on a 2 ( $E_2+$  and  $E_2-$ )  $\times$  2 (DHT+ and DHT-) design. In separate analyses, TP- and EB + DHTB-treated animals were compared to assess the effectiveness of our DHT+EB treatment as a substitute for TP treatment.

### Behavioral testing

During behavioral testing on the day of tissue punch or perfusion for immunocytochemistry, the following measures were recorded: mount latency (ML), latency from the introduction of the female to the first mount; intromission latency (IL), latency from introduction of the female to the first intromission; ejaculation latency (EL), latency from the first intromission to ejaculation; post-ejaculatory interval (PEI), latency from the first ejaculation to the first intromission of the second ejaculatory series; mount frequency-1 (MF1), number of mounts without vaginal insertion preceding the first ejaculation; intromission frequency-1 (IF1), number of mounts with penile insertion preceding the first ejaculation; mount frequency—total (MFT), the number of mounts per 30-min test; intromission frequency—total (IFT), the number of intromissions per 30-min test; and ejaculation frequency (EFT), the number of ejaculations per 30 min test.

### Statistical analysis of copulatory behaviors

Behavioral data from Experiments 1 and 2 were combined for statistical analysis since the hormonal treatments were identical in both experiments. First, the proportions of animals exhibiting mount, intromission, or ejaculation were analyzed with  $\chi^2$  tests, since many subjects, especially those in oil-, DHTB-, and EB-treated groups, failed to copulate. The behavioral measures described above were analyzed with a two-way ANOVA (presence or absence of  $E_2$  vs. DHT), followed by Student's *t* tests in cases of significant interactions ( $P < 0.05$ ). Latencies were analyzed only for animals that performed the respective behavior, and MF1, IF1, and PEI were analyzed only for animals that ejaculated. Student's *t* tests were used for comparison between TP- and EB+DHTB-treated animals.

### Experiment 1: Effects of T metabolites on MPOA DA content

#### Animals

Twenty-five sexually experienced males, weighing between 250 and 300 g, were used for this experiment. Animals were randomly assigned to five hormone treatment groups after initial copulation testing, as described above.

#### Preparation of brains

After behavioral testing, each subject was anesthetized with 0.5 ml sodium pentobarbital (50 mg/ml ip) and decapitated, and the brain was quickly removed and frozen at  $-80^\circ\text{C}$ . Using an 18-ga stainless steel punch tool, 500- $\mu\text{m}$ -thick bilateral punches were taken from each brain based on landmarks from the Paxinos and Watson (1998) atlas, placed in a 1.5-ml centrifuge tube and weighed (see Fig. 1a). Two hundred microliters of 0.01 N perchloric acid solution was added, and the tissue was dismembrated with a probe sonicator and centrifuged for 15 min at 10,000 rpm in centrifuge tubes with 0.22- $\mu\text{m}$  pore filters. Supernatant was immediately placed into a supercool freezer and maintained at a temperature of  $-80^\circ\text{C}$  until the time of assay.

#### HPLC-EC

Dopamine (DA) was assayed using HPLC-EC. Samples were loaded via a Rheodyne injector valve, which delivered a 500-nl volume to an LC-Packings Fusica C18 capillary column. Mobile phase, consisting of 30 mM citric acid, 50 mM sodium acetate, 0.027 mM  $\text{Na}_2\text{EDTA}$ , 0.25 mM octyl

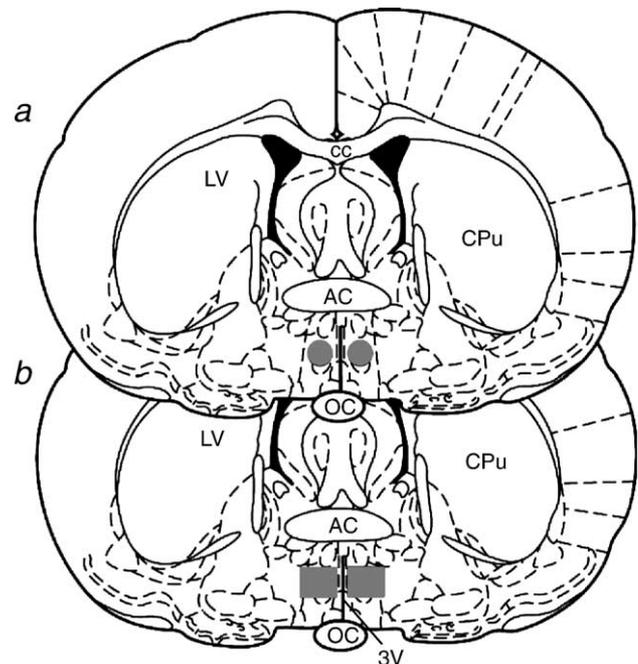


Fig. 1. (a) Approximate location of bilateral tissue punches. (b) Approximate location of bilateral counting field. Both adapted from Paxinos and Watson (1998). AC: anterior commissure; CC: corpus callosum; CPU: caudate putamen; LV: lateral ventricle; 3V: third ventricle.

sodium sulfate, and 2.5% acetonitrile (degassed and pH 3.8), was delivered by a Gilson model 307 pump operating at 0.5 ml/min. The Gilson pump was equipped with an Acurate flow splitter, which delivered 6  $\mu$ l/min to the column. Compounds were detected with an Antec microcell detector, using a glassy carbon-working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode.

Calibration curves, based on known amounts of external standards, were built each day of sample injection and assay. The amount of DA present in each tissue punch sample was determined from the calibration curve, using the Gilson Unipoint program.

#### Statistical analyses

The amount of DA was standardized for the tissue weight (DA in  $\mu$ g/ $\mu$ g of tissue). Tissue DA content data were analyzed using a two-way (presence or absence of E<sub>2</sub> vs. DHT) ANOVA, followed by Student's *t* tests ( $P < 0.05$ ). Student's *t* tests were used for comparison of TP- and EB + DHTB-treated animals.

#### Experiment 2: Effects of T metabolites on MPOA nNOS-ir

##### Perfusion and tissue preparation

Two hours after behavioral testing, each subject was deeply anesthetized with 1 ml sodium pentobarbital (50 mg/ml ip); the heart was exposed and the descending aorta clamped off with a hemostat. The right ventricle was injected with 0.1 ml of a heparin solution (20 U/0.1 ml), and the animal was perfused through the ascending aorta with 150 ml saline followed by 150 ml of freshly made 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in the perfusion fixative with 20% sucrose for cryoprotection at 4°C for 48 h. Coronal brain sections (40  $\mu$ m) were cut on a cryostat at -15°C, collected into PB containing 0.02% sodium azide, and stored at 4°C.

##### Immunocytochemistry

Free-floating slices were rinsed three times for five min each (3  $\times$  5 min) in 0.1 PB with Triton X-100 on a shaker. They were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min and again rinsed 3  $\times$  5 min. Brain slices were then incubated in normal goat serum (Vector Laboratories, Burlingame, CA) as a blocking agent for 30 min at room temperature. After 3  $\times$  5 rinses, the tissue was incubated in primary antibody (anti-nNOS raised in rabbit, at a dilution of 1:8000, Immunostar Corporation, Stillwater, MN.) for 36–48 h on a shaker at 4°C. At this time, the brain slices were rinsed (3  $\times$  5 min) and were incubated in biotinylated secondary antibody (anti-rabbit raised in goat, Vector Laboratories, Burlingame, CA; 1:200) for 2 h at room temperature. Three rinses followed; the tissue was incubated for 1 h. in Vectastain *Elite* ABC reagent (mixed according to manufacturer's recommendations) and rinsed 3  $\times$  5 min with an

additional 5-min rinse in 50 mM Tris buffer (TB, pH 7.6). For the chromogen reaction, the tissue was incubated in a 0.05% solution of 3',3'-diaminobenzidine tetrahydrochloride dihydrate (DAB, Sigma-Aldrich, St. Louis, MO) with 0.04% nickel chloride (NiCl) and 0.01% H<sub>2</sub>O<sub>2</sub> in TB. Slices were then rinsed in TB and were wet-mounted on gelatin-coated slides. The slides were allowed to dry overnight, then dehydrated (using sequential dips in 50%, 75%, 95%, and 100% ethanol), cleaned with Hemo-D, and coverslipped using Permount. One set of brain slices was incubated without primary antibody as a control.

#### Data analyses

Cells demonstrating nNOS immunoreactivity were counted in bilateral 0.4  $\times$  0.6 mm matched areas of the MPOA in representative brain slices from each animal based on the Paxinos and Watson (1998) atlas using an Olympus BX40 microscope and Image-Pro *Plus* program (Media Cybernetics, Silver Springs, MD; see Fig. 1b). The number of nNOS-ir cells per animal was expressed as number of nNOS-ir cells per mm<sup>2</sup>. Data were analyzed using a two-way ANOVA (presence or absence of E<sub>2</sub> vs. DHT), followed by Student's *t* tests ( $P < 0.05$ ). Student's *t* tests were used for comparison between TP- and EB+DHTB-treated animals.

## Results

### Copulatory behaviors

The proportion of animals exhibiting each of the copulatory behaviors is shown in Table 1. DHT [ $\chi^2_{(1)} = 6.29$ ,  $P = 0.012$ ] significantly increased the proportion of animals achieving ejaculation, compared with the expected proportion.

The effects of hormone treatment on mounts are shown in Table 2a. There was an interaction of E<sub>2</sub> and DHT [ $F(1,12) = 16.40$ ,  $P = 0.002$ ] and a main effect of DHT [ $F(1,12) = 16.88$ ,  $P < 0.001$ ] on MF1. Post hoc analyses revealed that in the absence of DHT, E<sub>2</sub> significantly reduced the number of mounts preceding ejaculation, while

Table 1  
Proportion of animals exhibiting copulatory behaviors

Hormone condition	DHT–	DHT+	Total
E <sub>2</sub> –	Mount: 9/11	Mount: 10/11	Mount: 19/22
	Intromission: 7/11	Intromission: 7/11	Intromission: 14/22
	Ejaculation: 1/11	Ejaculation: 5/11	Ejaculation: 6/22
E <sub>2</sub> +	Mount: 11/11	Mount: 9/11	Mount: 20/22
	Intromission: 8/11	Intromission: 8/11	Intromission: 16/22
	Ejaculation: 3/11	Ejaculation: 7/11	Ejaculation: 10/22
Total	Mount: 20/22	Mount: 19/22	Mount: 39/44
	Intromission: 15/22	Intromission: 15/22	Intromission: 30/44
	Ejaculation: 4/22*	Ejaculation: 12/22*	Ejaculation: 16/44

\* Significantly different from expected proportion ( $P < 0.05$ ).

Table 2a  
Effects of hormone treatment on mounts

Hormone condition		DHT–		DHT+			Total		
E <sub>2</sub> –	ML	285.6 ± 110.6	n = 9	ML	279.3 ± 110.6	n = 9	ML	282.4 ± 78.2	n = 18
	MF1	47 – –	n = 1	MF1	7.4 ± 3.5	n = 5	MF1	14.0 ± 4.1	n = 6
	MFT	21.4 ± 3.7	n = 11	MFT	8.2 ± 3.7	n = 11	MFT	14.8 ± 2.6	n = 22
E <sub>2</sub> +	ML	215.3 ± 100.0	n = 11	ML	29.2 ± 110.6	n = 9	ML	122.2 ± 74.6	n = 20
	MF1	17.0 <sup>a</sup> ± 4.3	n = 3	MF1	16.7 ± 2.8	n = 7	MF1	16.8 ± 2.6	n = 10
	MFT	21.4 ± 3.7	n = 11	MFT	16.0 ± 1.7	n = 11	MFT	18.7 ± 2.6	n = 22
Total	ML	250.4 ± 74.6	n = 20	ML	154.3 ± 78.2	n = 18	ML	202.4 ± 54.0	n = 38
	MF1	24.5 ± 4.3	n = 4	MF1	12.8 <sup>b</sup> ± 2.2	n = 12	MF1	15.8 ± 2.4	n = 16
	MFT	21.4 ± 2.6	n = 22	MFT	12.1 <sup>b</sup> ± 2.6	n = 22	MFT	16.7 ± 1.8	n = 44

<sup>a</sup> Significantly different from DHT– × E<sub>2</sub>– ( $P < 0.05$ ).

<sup>b</sup> Significantly different from DHT– ( $P < 0.05$ ).

in the presence of DHT, E<sub>2</sub> did not have a significant effect on that measure. In addition, there was a main effect of DHT on MFT [ $F(1,40) = 6.40$ ,  $P = 0.015$ ], indicating that the presence of DHT reduced the total number of mounts during the 30-min test.

The effects of hormone treatment on intromission are shown in Table 2b. There was a main effect of E<sub>2</sub> on IF1 [ $F(1,12) = 8.68$ ,  $P = 0.01$ ] and a main effect of DHT on IL [ $F(1,26) = 5.57$ ,  $P = 0.03$ ]. The presence of E<sub>2</sub> significantly increased the number of intromissions preceding ejaculation, while the presence of DHT shortened the latency to the first intromission.

The effects of hormone treatment on ejaculation and PEI are shown in Table 2c. There were significant main effects of DHT on EL [ $F(1,12) = 112.03$ ,  $P = 0.005$ ] and EFT [ $F(1,40) = 8.57$ ,  $P = 0.006$ ]. The presence of DHT was associated with shorter EL and with more ejaculations, compared with animals lacking DHT. No other behavioral measures were significantly different.

#### Comparison of behaviors between EB+DHTB- and TP-treated animals

Compared to EB+DHTB-treated animals, TP-treated animals had fewer mounts preceding ejaculation [ $t_{(14)} = 3.77$ ,  $P = 0.002$ ] and ejaculated sooner [ $t_{(14)} = 3.82$ ,  $P =$

0.002]. No other behavioral measures were significantly different (Table 3).

#### Experiment 1: Effects of T metabolites on MPOA tissue DA content

A two-way ANOVA detected a significant interaction of E<sub>2</sub> and DHT [ $F(1,15) = 6.31$ ,  $P = 0.02$ ] and a main effect of E<sub>2</sub> [ $F(1,15) = 19.47$ ,  $P < 0.001$ ] on tissue DA content, while the main effect of DHT was not significant [ $F(1,15) = 0.42$ ,  $P > 0.05$ ]. Post hoc tests revealed that E<sub>2</sub> significantly reduced the tissue DA content in the absence of DHT, but not in the presence of DHT (Fig. 2a). The MPOA tissue DA content of TP and DHTB+EB groups was not significantly different [ $t_{(8)} = 1.28$ ,  $P > 0.05$ ] (Fig. 2b).

#### Experiment 2: Effects of T metabolites on MPOA nNOS-ir

A two-way ANOVA detected a significant main effect of E<sub>2</sub> on the number of nNOS-ir cells [ $F(1,18) = 22.96$ ,  $P < 0.0001$ ], while the interaction [ $F(1,18) = 0.08$ ,  $P > 0.05$ ] and the main effect of DHT [ $F(1,18) = 0.58$ ,  $P > 0.05$ ] were not significant. Regardless of the presence or absence of DHT, E<sub>2</sub> significantly increased the number of nNOS-ir cells, resulting in nearly a threefold increase in nNOS-ir cells (Figs. 3a and 4). The MPOA nNOS-ir cell counts of the TP

Table 2b  
Effects of hormone treatment on intromissions

Hormone condition		DHT–		DHT+			Total		
E <sub>2</sub> –	IL	589.6 ± 138.3	n = 7	IL	295.0 ± 138.3	n = 7	IL	442.3 ± 97.8	n = 14
	IF1	5.0 – –	n = 1	IF1	6.0 ± 1.4	n = 5	IF1	5.8 ± 1.7	n = 6
	IFT	3.5 ± 1.6	n = 11	IFT	4.9 ± 1.6	n = 11	IFT	4.2 ± 1.1	n = 22
E <sub>2</sub> +	IL	520.3 ± 129.4	n = 8	IL	182.9 ± 129.4	n = 8	IL	351.6 ± 91.5	n = 16
	IF1	13.7 ± 1.7	n = 3	IF1	8.9 ± 1.1	n = 7	IF1	10.3 <sup>b</sup> ± 1.0	n = 10
	IFT	6.5 ± 1.6	n = 11	IFT	8.2 ± 1.6	n = 11	IFT	7.4 ± 1.1	n = 22
Total	IL	552.6 ± 94.7	n = 15	IL	235.2 <sup>a</sup> ± 94.77	n = 15	IL	393.9 ± 67.0	n = 30
	IF1	11.5 ± 1.7	n = 4	IF1	7.4 ± 0.9	n = 12	IF1	8.6 ± 1.0	n = 16
	IFT	5.0 ± 1.1	n = 22	IFT	8.5 ± 1.12	n = 22	IFT	5.8 ± 0.8	n = 44

<sup>a</sup> Significantly different from DHT– ( $P < 0.05$ ).

<sup>b</sup> Significantly different from E<sub>2</sub>– ( $P < 0.05$ ).

Table 2c  
Effects of hormone treatment on Ejaculation and postejaculatory Interval (PEI)

Hormone condition	DHT–			DHT+			Total		
E <sub>2</sub> –	EL	1498 – –	<i>n</i> = 1	EL	525.6 ± 128.0	<i>n</i> = 5	EL	687.7 ± 156.8	<i>n</i> = 6
	EFT	0.09 ± 0.19	<i>n</i> = 11	EFT	0.64 ± 0.19	<i>n</i> = 11	EFT	0.36 ± 0.13	<i>n</i> = 22
	PEI	– – –	<i>n</i> = 0	PEI	824.4 ± 128.3	<i>n</i> = 5	PEI	824.4 ± 128.3	<i>n</i> = 5
E+	EL	1393.7 ± 165.3	<i>n</i> = 3	EL	1080.7 ± 108.2	<i>n</i> = 7	EL	1174.6 ± 98.8	<i>n</i> = 10
	EFT	0.27 ± 0.19	<i>n</i> = 11	EFT	0.82 ± 0.19	<i>n</i> = 11	EFT	0.55 ± 0.13	<i>n</i> = 22
	PEI	510.5 ± 202.9	<i>n</i> = 2	PEI	605.4 ± 108.5	<i>n</i> = 7	PEI	595.1 ± 115.0	<i>n</i> = 9
Total	EL	1419.8 ± 165.3	<i>n</i> = 4	EL	849.4 <sup>a</sup> ± 83.8	<i>n</i> = 12	EL	992.0 ± 92.1	<i>n</i> = 16
	EFT	0.18 ± 0.13	<i>n</i> = 22	EFT	0.73 <sup>a</sup> ± 0.13	<i>n</i> = 22	EFT	0.45 ± 0.09	<i>n</i> = 44
	PEI	510.5 ± 202.9	<i>n</i> = 2	PEI	696.7 ± 84.0	<i>n</i> = 12	PEI	644.9 ± 87.8	<i>n</i> = 14

<sup>a</sup> Significantly different from DHT– (*P* < 0.05).

and DHTB+EB groups were not significantly different [ $t_{(8)} = 1.41$ , *P* > 0.05] (Fig. 3b).

## Discussion

E<sub>2</sub> and DHT, the two main metabolites of T, differentially affected sexual behavior, tissue content of DA (a measure of stored neurotransmitter), and nNOS-ir in the MPOA of male rat castrates. In both experiments, copulatory behavior observed within the various hormone treatment groups was similar to that observed in some previous studies (Baum and Vreeburg, 1973; Davidson, 1969; reviewed in Hull et al., 2002), although oil-, DHTB-, and EB-treated groups exhibited somewhat more robust sexual activity than in an earlier study from this lab (Putnam et al., 2003). Treatment with DHTB or EB alone was not sufficient to maintain ejaculation in most animals, although the majority of them intromitted and mounted. Combined DHTB and EB treatment successfully maintained full copulatory ability in the majority of animals, although slightly less effectively than TP treatment.

Statistical analyses indicate that both E<sub>2</sub> and DHT contributed to the maintenance of normal mounting

behavior. The presence of either hormone reduced the number of mounts, resulting in more efficient copulation. IF1 was increased by E<sub>2</sub>, compared with animals lacking E<sub>2</sub>. This increase in intromissions preceding ejaculation is reminiscent of the greater number of preejaculatory intromissions in gonadally intact males, compared to recent castrates (Davidson, 1966). Thus, T, via its metabolite E<sub>2</sub>, may prevent “premature ejaculation” and may thereby increase the number of sperm in the ejaculate, facilitate sperm transport, and promote a progestational state in the female (Toner and Adler, 1986; Toner et al., 1987).

Ejaculation appears to be maintained by DHT, as seen in its effect on the proportion of animals that ejaculated. Of those animals that ejaculated, the latency to ejaculate and the total number of ejaculations were maintained by DHT, with shorter latencies to ejaculate and more ejaculations occurring in animals with DHT.

In Experiment 1, the higher MPOA tissue DA content in oil-treated castrates is consistent with a previous report that castrates had higher DA content than either TP-treated or gonadally intact males (Du et al., 1998). Du et al. suggested that the higher DA content in castrates resulted, at least in part, from decreased release, since extracellular DA levels

Table 3  
Behavioral comparison between EB + DHTB- and TP-treated animals

Hormone condition	Mount	ML		MF1		MFT	
EB + DHTB	9/11	29.2 ± 110.6	<i>n</i> = 9	16.7 ± 2.8	<i>n</i> = 7	16.0 ± 1.7	<i>n</i> = 11
TP	9/11	68.0 ± 26.6	<i>n</i> = 10	4.6* ± 0.9	<i>n</i> = 9	11.5 ± 2.0	<i>n</i> = 11
	Intromission	IL		IF1		IFT	
EB + DHTB	8/11	182.9 ± 129.4	<i>n</i> = 8	8.9 ± 1.1	<i>n</i> = 7	8.2 ± 1.6	<i>n</i> = 11
TP	10/11	184.5 ± 77.3	<i>n</i> = 10	7.6 ± 0.8	<i>n</i> = 9	12.4 ± 2.4	<i>n</i> = 11
	Ejaculation	EL		EFT		PEI	
EB + DHTB	7/11	1080.7 ± 108.2	<i>n</i> = 7	0.8 ± 0.19	<i>n</i> = 11	605.4 ± 108.5	<i>n</i> = 7
TP	9/11	488.1* ± 102.0	<i>n</i> = 9	1.5 ± 0.3	<i>n</i> = 11	605.8 ± 79.4	<i>n</i> = 9

Comparison between DHTB + EB- and TP-treated animals.

\* Significantly different from DHTB + EB-treated animals.

## Tissue DA Content

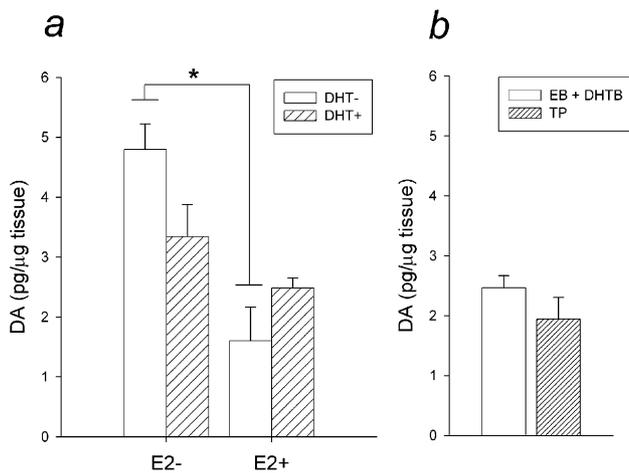


Fig. 2. Tissue content of DA in the MPOA. (a) Comparison among EB- and/or DHTB-treated animals. (b) Comparisons between DHTB + EB- and TP-treated animals. \* $P < 0.05$ .

were lower in castrates and since amphetamine administration led to greater release of DA in castrates. Therefore, there was more DA available in castrates, but an impaired release mechanism resulted in lower extracellular levels. In the present study, there was a significant interaction between  $E_2$  and DHT, with  $E_2$  significantly reducing DA content in the absence of DHT, but not in animals receiving DHT. Apparently,  $E_2$  maintains DA storage (or release) at near normal levels. DHT seems to attenuate the effects of the lack of  $E_2$ , possibly by maintaining normal sensory inputs through the olfactory system and the penis, which may acutely increase DA release in the MPOA (Dominguez and Hull, 2001; Triemstra et al., in press), and thereby decrease DA stores.

In Experiment 2, nNOS-ir cells were maintained by  $E_2$ . There were more nNOS-ir cells in the MPOA of animals with  $E_2$  (EB- and EB + DHTB-treated groups), compared to those lacking  $E_2$  (oil- and DHTB-treated groups). In fact, the numbers of nNOS-ir cells observed in this study were almost identical to those in our previous study, in which long-term castrates and TP-treated or intact animals were compared without behavioral testing (Du and Hull, 1999). In contrast to the effects of  $E_2$ , there seems to be virtually no contribution of DHT to this measure. This contradicts the finding by Singh et al. (2000), which found that nNOS-ir, NOS activity, and nNOS mRNA expressions were increased by castration; those increases were attenuated by either T or DHT administration. This discrepancy may be partially attributable to differences in the antibody used and in the brain area examined. Singh et al. studied a somewhat more posterior area than did the present experiment and reported that few NOS-ir neurons were observed, in contrast with the more robust staining in the present study. Others have found the decrease in nNOS-ir with castration, using the same

antibody as we used in this and previous studies (Du and Hull, 1999; Hadeishi and Wood, 1996; Scordalakes et al., 2002). On the other hand, we did not detect a significant change in nNOS protein levels in Western blots using the same polyclonal antibody as in the present experiment (unpublished observation). Thus, the exact nature of changes in nNOS induced by hormonal treatment is not clear at this point.

Although the doses that were used in these experiments provided supra-physiological levels of hormones, they were the same as those used in our previous studies and enabled us to compare the results of this and those previous studies. In addition, these doses were maximally effective in maintaining or restoring copulation in castrated males (Feder et al., 1974; Larsson et al., 1973).

It is possible that the changes in MPOA tissue DA content and nNOS-ir observed are secondary to the effects of hormones on copulatory behaviors, since the animals in this study underwent behavioral testing prior to sacrifice. However, we have previously observed changes in these measures that are consistent with the current study, even when no behavioral testing was conducted immediately before sacrifice (Du and Hull, 1999; Du et al., 1998). Thus, it seems likely that the changes in MPOA tissue DA content and nNOS-ir were directly dependent on our hormone treatment, and not on behavioral changes induced by hormone treatment.

This study extends our previous findings on the effects of T on MPOA tissue DA content (Du et al., 1998) and on MPOA nNOS-ir (Du and Hull, 1999). The current data suggest that  $E_2$  mediates the effects of T on MPOA DA content, with some attenuation by DHT. The effects of T on nNOS also seem to be mediated by  $E_2$ , with virtually no contribution by DHT. These results are not surprising, given the well-documented importance of estrogenic stimulation in the MPOA for male copulatory behaviors (Christensen

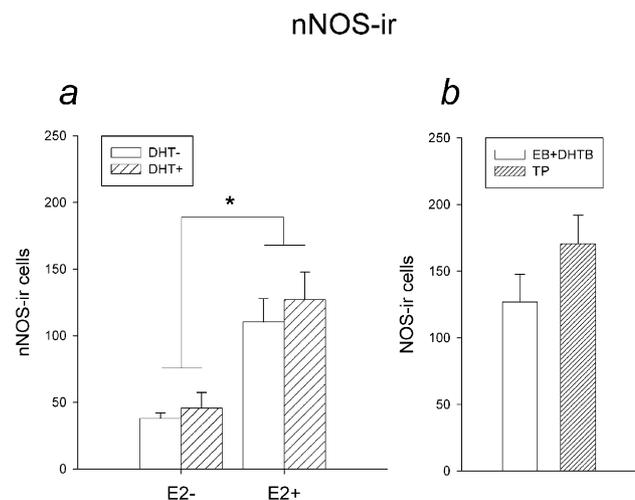


Fig. 3. Number of nNOS-ir positive cells in the MPOA. (a) Comparison among EB- and/or DHTB-treated animals. (b) Comparisons between DHTB + EB- and TP-treated animals. \* $P < 0.05$ .

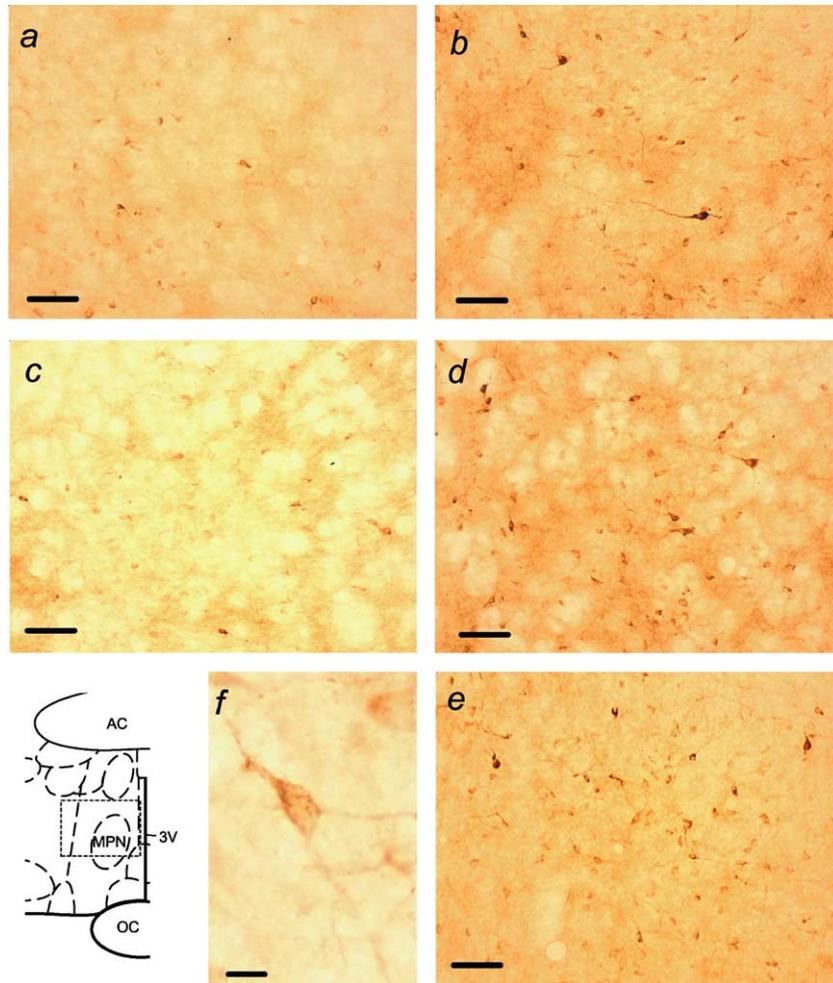


Fig. 4. Representative photomicrographs of the MPOA nNOS staining. (a) Oil-, (b) EB + DHTB-, (c) DHTB-, (d) EB-, and (e) TP-treated animals. All sections shown were processed in the same batch and photographed under identical conditions. Scale bar = 100  $\mu$ m (a, b, c, d, e). (f) A photomicrograph of a representative nNOS-ir cell in the MPOA at higher magnification. Scale bar = 10  $\mu$ m (f).

and Clemens, 1974; Clancy et al., 1995, 2000; Davis and Barfield, 1979; Lisk and Bezier, 1980; Vagell and McGinnis, 1997).

In addition to identifying the relative contribution of T metabolites in mediating the effects of T on tissue DA content and nNOS-ir, the current study augments our previous work on the effects of T metabolites on extracellular DA levels in the MPOA (Putnam et al., 2003). We previously observed higher basal extracellular DA levels in castrates with  $E_2$  (EB, TP, and EB+DHTB groups) than in those without (oil and DHTB groups). In contrast, we observed lower DA tissue content in EB-treated animals, compared to oil-treated animals. We also observed slightly lower DA tissue contents in EB+DHTB-treated animals compared to DHT-treated animals, although that difference was not statistically significant. In other words,  $E_2$  appears to increase extracellular DA and decrease tissue DA content. This suggests that  $E_2$  affects a DA-releasing mechanism in the MPOA, contributing to the changes in intra- and extracellular DA levels and the behavioral deficits seen in these animals.

Our rationale for examining nNOS-ir was based on our previous findings that NO facilitates both basal and female-stimulated DA release in the MPOA (Lorrain and Hull, 1993; Lorrain et al., 1996) and that T alters nNOS-ir (Du and Hull, 1999). Furthermore, we and others have reported copulatory impairment following administration of NOS antagonists into the MPOA (Lagoda et al., 2004; Sato et al., 1998a) or with systemic or intracerebroventricular administration (Benelli et al., 1995). Similarly, NO appears to facilitate female sexual behavior (Chu and Etgen, 1999; Mani et al., 1994). The data from this study, combined with our previous studies, suggest that (1)  $E_2$ , synthesized from T, alters nNOS in the MPOA, resulting in increased NO production; and (2) this increase in NO production in turn increases DA release, resulting in decreased intracellular storage and enhanced sexual behavior. DHT, most likely by enhancing the sensory inputs, can attenuate some but not all of the changes associated with lack of estrogenic stimulation in the MPOA (see Fig. 5). For example, DHT is known to be the major T metabolite that maintains erections and penile structures (Meisel et al., 1984) and contributes to restoration

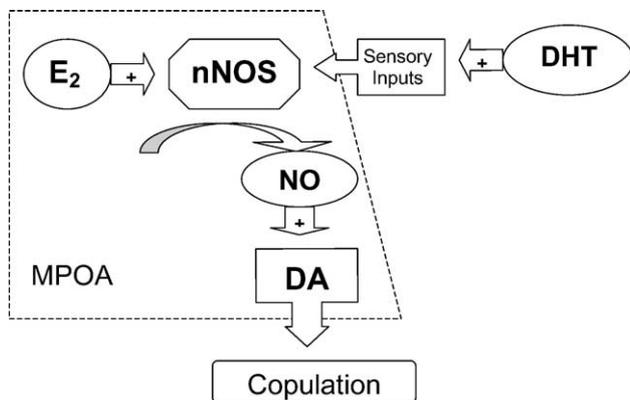


Fig. 5. Schematic representation of effects of E<sub>2</sub> and DHT on nNOS, NO, DA, and copulation. E<sub>2</sub> stimulates nNOS in the MPOA, thereby increasing NO production, which facilitates DA release and copulation. DHT, by its effects outside the MPOA, enhances sensory inputs to the MPOA, which contributes to acute stimulation of nNOS in the MPOA.

of copulation when implanted in the medial amygdala, an important structure for processing of chemosensory input (Baum et al., 1982). Normal basal DA levels, maintained by the effects of E<sub>2</sub> on nNOS, appear to be important for the initiation of copulation, while the expression of full copulatory behaviors depends on the female-stimulated DA release, which requires DHT (Putnam et al., 2003).

The data currently available do not exclude the possibility that T affects MPOA DA levels in an nNOS-independent manner. In fact, it is quite conceivable that such steroidal modulation exists, given the profound influence of gonadal steroids on the development of hypothalamic DA cells (Simerly, 1989; Simerly et al., 1985a,b, 1997) and the report that at least some of these DA cells concentrate E<sub>2</sub> (Heritage et al., 1980; Sar, 1984). The co-localization of steroid receptors in A12 DA cells has also been reported (Batailler et al., 1992). Furthermore, gonadal steroids regulate tyrosine hydroxylase (TH, the rate-limiting enzyme of DA synthesis) expression in A13 DA cells in the zona incerta (Sanghera et al., 1991). However, we did not observe any changes in the number of TH-ir cells in the periventricular nucleus (PeVN) with castration (Du and Hull, 1999). Currently, we are not aware of any report of such direct regulation of hypothalamic DA cells by gonadal steroids in adult male rodents.

The effects of E<sub>2</sub> on MPOA nNOS have implications beyond its influence on DA levels. For example, NO in the MPOA is known to regulate GnRH release via the NO/cGMP pathway in rats (Pu et al., 1998). Such effects suggest that NO may be a key modulator in the integration of sensorimotor and neuroendocrine pathways involved in regulation of male copulation. In other words, NO production stimulated by sensory inputs from a female conspecific may trigger, not only DA release and subsequent behavioral outputs, but also endocrine responses that accompany copulation as well (see Amistislvskaya and Popova, 2004, for example). In addition, NO is known to inhibit aromatase (Snyder et al., 1996), which converts T

to E<sub>2</sub>. The regulation of nNOS by E<sub>2</sub>, and its influence on the hypothalamus–pituitary–gonadal axis, may function as a negative feedback mechanism on gonadal steroid secretion.

In summary, we found that both E<sub>2</sub> and DHT contribute to the maintenance of copulatory behavior. DHT increased the proportion of animals that ejaculated, and both metabolites decreased the numbers of mounts preceding ejaculation. However, E<sub>2</sub> alone increased the numbers of intromissions preceding ejaculation and the total number of intromissions, and only DHT significantly decreased the latency to ejaculate and the numbers of ejaculations, although there was a trend for an increase in numbers of ejaculations by E<sub>2</sub>. In addition, these data, along with our previous findings (Lorrain and Hull, 1993; Lorrain et al., 1996; Putnam et al., 2003), suggest that E<sub>2</sub> regulates nNOS in the MPOA of male rats, which in turn regulates MPOA DA levels. NO in the MPOA may serve as an important messenger in the integration of sensorimotor and neuroendocrine pathways involved in regulation of male reproductive processes. DHT, an androgenic metabolite of T, had relatively little influence on nNOS or DA levels in the MPOA. The facilitative effects of DHT on copulation may be mediated in part by its permissive effects on chemosensory and genital sensory pathways that increase DA release in response to a female (Dominguez and Hull, 2001; Putnam et al., 2003; Triemstra et al., in press).

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