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5α-Androstane-3β,17β-diol Binds to Androgen and Estrogen Receptors Without Activating Copulatory Behavior in Female Rats

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DOERING, C. H. AND B. A. GLADUE. *5α-Androstane-3β,17β-diol binds to androgen and estrogen receptors without activating copulatory behavior in female rats.* PHARMAC. BIOCHEM. BEHAV. **16**(5) 837–840, 1982.—The ability of the androgen metabolite 5α-androstane-3β,17β-diol (3β-A-diol) to facilitate copulatory behavior was assessed directly in adult ovariectomized rats. Neither the highest dosage of 5 mg/day for three days, nor 2 mg/day for 15 days could induce lordosis behavior in females that displayed typically high lordosis quotients with low dosages of estradiol (E). Furthermore, prolonged administration of 5α-dihydrotestosterone (DHT) induced a low but significant level of male-typical mounting behavior in females, whereas 3β-A-diol administered for 20 days (2 mg/day) had no effect on mounting behavior. However, this reduced androgen metabolite did compete moderately well for DHT and E binding sites on androgen and estrogen receptors respectively in hypothalamic cytosol preparations. We conclude that in spite of its ability to bind to these receptors in the brain 3β-A-diol, a major metabolite of DHT, is totally inert with respect to sexual behavior.

Lordosis Mounting behavior Hypothalamus Estrogen receptors Androgen receptors 5α-Androstane-3β,17β-diol

FEMININE sexual response in rats is thought to be mediated by estrogen receptors in the brain. Very small quantities of estradiol-17β (E) readily induce the lordosis behavior pattern in females. In addition to its effect on masculine sexual behavior, testosterone (T) also can induce the lordosis response presumably by its metabolic conversion in the brain to E [10,17]. The observation of Beyer, Morali, and Cruz [5] that 5α-dihydrotestosterone (DHT) can stimulate the lordosis response does not seem to fit the same mechanism of action, since DHT neither binds to the estrogen receptor nor can it be metabolized to an estrogen. However, the finding that the DHT metabolite 5α-androstane-3β,17β-diol (3β-A-diol) binds to brain estrogen receptor [9,16] led Sodersten and Gustafsson [14] to suggest that DHT can induce lordosis behavior via its reductive metabolite. We have examined this possibility directly by studying the behavioral responses of female rats to the administration of various dosages of 3β-A-diol and by reassessing the relative binding affinity of 3β-A-diol to estradiol and androgen receptors of the hypothalamus.

METHOD

Adult Long-Evans female rats obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) were maintained by the Division of Laboratory Animal Resources of the SUNY at Stony Brook Health Sciences Center. They were housed in wire-mesh metal cages (15×30×34 cm), three animals per cage, with food and water ad lib, at 23°C in a reversed dark/light cycle (12/12 hours; lights off at 1000 hr). Ovariectomies under ketamine hydrochloride anesthesia were performed with a bilateral flank approach. Wounds were sealed with silk and dissolvable sutures, followed by wound clips on the exterior incision.

Hormones were dissolved in sesame oil (Laboratory Grade, Fisher Scientific Co.) and administered by intramuscular injection of 0.1 ml each day. The dosage of 5 and 2 mg of 3β-A-diol dipropionate per 0.1 ml of oil could be brought into solution only by heating immediately before injection. Steroids were obtained from Steraloids, Inc. (Wilton, NH); diethylstilbestrol, from Sigma Chemical Co. (St. Louis, MO); Sephadex LH-20, from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were reagent grade, or highest purity available; water for making buffer was either triple-distilled or HPLC-grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI).

Receptor Binding Studies

Adult female Long-evans rats were ovariectomized at least three days before receptor assays. Animals were anesthetized with sodium pentobarbital before decapitation. Brains were excised, immediately placed on ice, and bathed

with ice-cold buffer (see below). Hypothalamic-preoptic areas (HYP-POA) were cut by these delineations: laterally by the hypothalamic sulci, posteriorly by mammillary bodies, 2 mm anterior to the optic chiasm, and dorsally by a depth of 2 mm along the border of the thalamus. Tissue was kept chilled, weighed, and homogenized by hand-held loose-fitting Teflon pestle (Kontes Glass Co., Vineland, NJ) in pH 7.4 buffer (1:3 w/v) consisting of 10mM sodium-potassium phosphate, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol. The homogenate was first centrifuged at 0–2°C for 10 min at 1500 ×g. Cytosol was prepared by recentrifugation of the supernatant at 0–2°C for 60 min at 105,000 ×g in a swinging-bucket rotor. To improve receptor stability [7], cytosol was diluted 9:1 (v/v) with 200 mM Na_2MoO_4 in homogenizing buffer (final molybdate concentration in cytosol was 20 mM). All solutions were kept ice-cold and most operations performed at 4°C in a cold room. Cytosol prepared in this manner was used for both estrogen and androgen receptor assays.

Estrogen receptor assay. With few exceptions we followed the method of Ginsburg, Greenstein, MacLusky, Morris and Thomas [8]. We reconfirmed the critical parameters of incubation and separation conditions. As a routine, 0.20 ml of HYP-POA cytosol with molybdate and containing 0.5 to 0.8 mg total protein were incubated with 0.10 ml of homogenizing buffer containing 0.30 pmoles of (2,4,6,7,16,17-^{3}H)-estradiol-17β (New England Nuclear Corp., Boston, MA), specific activity 130.0 Ci/mmole, for a final concentration of 1×10^{-9}M. For determining nonspecific binding, some tubes also contained 30 pmoles of unlabeled estradiol-17β for a final concentration of 1×10^{-7} M. Tubes were incubated for 10 min at 30°C in a shaking waterbath and returned to ice. Separation of free from bound radioligand was effected in a cold room at 4°C by chromatography on 0.5×7 cm columns of Sephadex LH-20. Of the incubation mixture, 0.25 ml was transferred to the column and rinsed in with 0.10 ml of buffer. After standing for 30 min at 4°C, 0.20 ml of buffer was added and the effluent discarded. Macromulecular-bound radioligand was eluted into scintillation vials with 0.60 ml of buffer and counted with 4.5 ml of Hydrofluor scintillation fluid (National Diagnostics, Parsippany, NJ) to an error of less than 2% with 95% confidence. Counting efficiency was determined with internal standards in parallel tubes under identical conditions.

For the competition study, 10^{-9} M ^{3}H-estradiol was incubated as described above in the presence of 10^{-8} or 10^{-7} M competing substance.

Androgen receptor assay. We followed the method of Barley, Ginsburg, Greenstein, MacLusky and Thomas [2] after checking the incubation and separation conditions. Generally, 0.20 ml of HYP-POA cytosol with molybdate and containing 0.6 to 0.9 mg total protein were incubated with 0.10 ml of buffer containing 0.60 pmoles of (1,2,3,5,6,7-^{3}H)-5α-dihydrotestosterone (New England Nuclear Corp.), specific activity 131.0 Ci/mmole, for a final concentration of 2×10^{-9} M. For determining nonspecific binding, some tubes also contained 60 pmoles of unlabeled 5α-dihydrotestosterone for a final concentration of 2×10^{-7} M. Tubes were incubated for 17–19 hours at 0°C in a shaking waterbath. Separation of free from bound radioligand and radioactivity counting was performed exactly as described above for estrogen receptor assay.

For the competition study, 2×10^{-9} M ^{3}H-DHT was incubated as described above in the presence of 2×10^{-8} or 2×10^{-7} M competing steroid.

Behavioral studies

Feminine sexual behavior. Females to be tested for lordosis behavior were injected with 0.50 mg of progesterone four hours before start of the test. Three hours after onset of the dark phase and in dim red light, a sexually vigorous stud male was placed in a Plexiglas testing arena (44×57×50 cm, w×l×h) containing bedding material and 5 min later the female test animal was introduced to the stud male. The number of lordotic responses to mounts was recorded for the first 10 mounts received by each test animal. Lordosis is defined as the ventral arching of the back with dorsal elevation of the head and of the perineal region and with lateral tail deflection. The lordosis quotient (LQ) is the ratio of lordosis responses to mounts received ×100.

Masculine sexual behavior. Adult female rats display mounting behavior, but rarely intromission behavior patterns. Consequently, we recorded only mounting behavior. A mount is described as the animal grasping the stimulus female's flanks with its forepaws accompanied by vigorous pelvic thrusting to her ano-genital region. Between 3 and 6 hr after onset of the dark phase in dim red light, the test female was placed into the testing arena described above. Five minutes later, at the start of the test period, a highly proceptive and receptive stimulus female was introduced to the test female. The number of times the test female mounted the stimulus female during the 20-minute test period (=mount frequency) was recorded. Stimulus females were prepared by ovariectomy and implantation of a 4-mm length of Silastic capsule (1.57 mm i.d., 3.18 mm o.d. Medical Grade Tubing, Dow Corning, Midland, MI, Cat. No. 602-285) filled with crystalline estradiol. Four hours before their use, stimulus females received 0.5 mg of progesterone.

For this experiment, 12 ovariectomized females were injected daily for 40 days with 0.25 or 1 mg of DHT propionate and mount frequency was assessed in five weekly tests. After three weeks of no treatment, these females were tested for lordosis response to 2 μg of E benzoate administered for two days. After another 1-week period of no treatment, 2 mg of 3β-A-diol dipropionate was administered daily for 20 days. Mount frequency was assessed in three weekly tests; lordosis response, two days after the second mount frequency test.

RESULTS AND DISCUSSION

Receptor Binding Study

The data in Tables 1 and 2 confirm previous findings of the moderate binding affinity of 5α-androstane-3β,17β-diol to rat hypothalamic estrogen receptors [9,16] and androgen receptors [2,11]. One report of the relative binding affinity of this metabolite for estrogen binding sites was based on competition with a broad range of concentrations [16]. Others have obtained the dissociation constant by Scatchard analysis of 3β-A-diol for hypothalamic estrogen receptor: a value of 3.3×10^{-8} M compared to 1.3×10^{-10} M for E [9]. Our data were derived from the use of the adult female rats, two concentrations of competitors, and by methods that differ from those used by Vreeburg *et al.* [16] and Hannouche *et al.* [11]. Nevertheless, the results are essentially the same: The DHT metabolite 3β-A-diol competes for estradiol binding sites much better than either DHT itself or the 3α-A-diol, although it competes much less than the estrogens estradiol and diethylstilbestrol (Table 1). In the case of the androgen receptor, the apparent binding affinity of 3β-A-diol is similar

TABLE 1

LIGAND SPECIFICITY OF ESTROGEN RECEPTOR IN HYP-POA CYTOSOL PREPARATIONS OF OVARIECTOMIZED ADULT RATS

Unlabeled Substance†	Competing Efficiency* at 10 nM‡	at 100 nM‡
E	91	100
DES	96	99
DHT	0	2
3α-A-diol	−2	2
3β-A-diol	1	30

Radioligand was 1 nM ^{3}H-E. (Means of duplicate incubations.)

*Values of competing efficiency were computed as suggested by Atger *et al.* [1]. Binding in the presence of 100 nM unlabeled E arbitrarily has been defined as 100.

†E, estradiol-17β; DES, diethylstilbestrol; DHT, 5α-dihydrotestosterone; 3α-A-diol, 5α-androstane-3α,17β-diol; 3β-A-diol, 5α-androtane-3β,17β-diol.

‡Final concentration of competing substance in the incubation mixture.

TABLE 2

LIGAND SPECIFICITY OF ANDROGEN RECEPTOR IN HYP-POA CYTOSOL PREPARATIONS OF OVARIECTOMIZED ADULT RATS

Unlabeled Steroid†	Competing Efficiency* at 20 nM‡	at 200 nM‡
DHT	84	100
3α-A-diol	3	51
3β-A-diol	40	84
T	77	—
E	29	80
Progesterone	12	—
Corticosterone	—	−1

Radioligand was 2 nM ^{3}H-DHT. (Means of 2–4 replicate incubations.)

*See comparable footnote to Table 1. Binding in the presence of 200 nM unlabeled DHT arbitrarily has been defined as 100.

†For abbreviations see Text or Table 1.

‡Final concentration of competing steroid in the incubation mixture.

to that of estradiol and both affinities are lower than those of DHT and T (Table 2). The 3α-epimer competes poorly for androgen receptor binding sites.

When relatively large amounts of DHT are administered to rats, the concentrations of the 3β-A-diol metabolite could well become high enough for binding to hypothalamic estrogen receptor and for subsequent nuclear translocation to cell nuclei. The observation of such translocation of estradiol receptor by 3β-diol was recently reported by Kreitmann and Bayard [12] for breast cancer cells and by Thieulant, Samperez and Jouan [15] for rat pituitary. The work of Rochefort and Garcia with calf and rat uterus supports the same conclusion [6,13].

Behavioral Studies

The effect of 3β-A-diol on female sexual behavior has heretofore not been assessed directly. The administration to ovariectomized females (n=7 per group) of either 1 mg or 5 mg per day of the dipropionate derivative did not facilitate lordosis behavior four hours after receiving progesterone (LQ=0; 0% of animals responding). After this preliminary assessment, 2 mg per day of 3β-A-diol dipropionate was administered for 15 days to another group of rats (n=12). Again no lordotic response was seen (LQ=0; 0% of animals responding), although these same rats displayed high levels of lordosis behavior (LQ=98; 100% of animals responding) with 2 μg E benzoate. These observations appear to negate the mechanism suggested by Sodersten and Gustafsson [14] for the induction of lordosis behavior by DHT. The androgen metabolite 3β-A-diol is not involved in the activation of lordosis behavior.

Since 3β-A-diol binds fairly well to hypothalamic androgen receptors (relatively better than to estrogen receptors) we tested also for any effects on masculine sexual behavior in ovariectomized female rats. While the prolonged administration of DHT propionate typically yielded mount frequencies in six out of 12 females, 2 mg/day of 3β-A-diol dipropionate administered for three weeks did not induce mounting behavior in these same females (Table 3). The behavioral effect of the 3β-A-diol thus is similarly absent as

TABLE 3

MASCULINE BEHAVIORAL RESPONSE IN ADULT FEMALE OVARIECTOMIZED RATS*

Treatment†	Mount Frequency	Animals Responding (%)
DHTP, 0.25 or 1 mg/day for 40 days (mean of 5 weekly tests)	3.5 ± 0.78	47
3β-A-diol diprop., 2 mg/day for 20 days (mean of 3 weekly tests)	0.9 ± 0.56‡	11§
No treatment for 13 days (single test)	0	0
Oil vehicle alone for 14 days (mean of 2 tests)	0.4 ± 0.29	8

*N=12 animals. Shown are Means ± S.E.M.

†DHTP, 5α-dihydrotestosterone propionate; 3β-A-diol diprop., 5α-androstane-3β,17β-diol dipropionate.

‡Significantly different from DHTP treatment, $p=0.05$, but not different from oil control treatment (Wilcoxson Sign Test).

§Significantly different from DHTP treatment by proportion test ($z=1.96$; $p=0.05$, two tailed), but not different from oil control group.

that of the 3α-epimer originally described by Beyer, Larsson, Perez-Palacios and Morali [4]. The observed facilitation of mounting behavior by DHT, therefore, cannot be mediated by its 3-reduced metabolites. Although in combination with DHT 3β-A-diol appears to activate masculine behavior patterns in castrated male rats [3], it is not clear by what mechanism this effect is achieved since the diol does not activate the behavior by itself in females.

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