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# Prenatal Antiandrogen Feminizes Behavioral but not Neurochemical Response to Estrogen

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DOHANICH, G. P., J. A. WITCHER AND L. G. CLEMENS. *Prenatal antiandrogen feminizes behavioral but not neurochemical response to estrogen.* PHARMACOL BIOCHEM BEHAV **23**(3) 397–400, 1985.—In ovariectomized female rats, estrogen activates high levels of female sexual behavior and induces an increase in the number of cholinergic muscarinic binding sites in the medial basal hypothalamus, an area known to regulate this behavior. Male rats normally show low levels of female sexual behavior and no alteration in muscarinic binding in response to estrogen treatment. An experiment was conducted to determine if a prenatal treatment that feminizes the sexual behavior of male rats would also establish the potential for estrogen to increase muscarinic binding in the male hypothalamus. Results indicate that prenatal exposure to the antiandrogen, flutamide, enhanced estrogen-activated female sexual behavior in male rats but failed to reverse the inability of estrogen to increase muscarinic binding in the medial basal hypothalamus.

Prenatal antiandrogen Estrogen Female sexual behavior in male rats

ANDROGENS present during prenatal and early postnatal life exert a powerful organizational effect on specific brain areas that control sexual reproduction. One consequence of perinatal exposure to androgen is that normal male rats are often not as responsive to estrogen in adulthood as female rats. For example, estrogen regimens that activate a high level of female sexual behavior in female rats are usually less effective in activating this same behavior in males [15]. Male rats deprived of endogenous androgen by prenatal exposure to antiandrogens, however, display elevated levels of female sexual behavior as adults following estrogen treatment [9].

The ventromedial hypothalamus (VMH) has been shown to be the principal site at which estrogen acts to initiate sexual behavior in female rats [18]. Although the mechanism by which estrogen activates sexual behavior has not been identified, its ability to regulate neurotransmission may be an important feature. Appropriately, the number of cholinergic muscarinic binding sites in the medial basal hypothalamus (MBH), and specifically in the VMH, of ovariectomized female rats is increased significantly following treatment with estradiol [7, 14, 16, 17]. This increase in muscarinic binding sites may have behavioral implications since cholinergic activity appears to contribute to the regulation of lordosis, an estrogen-dependent sexual behavior [2–6].

Male rats, which display low levels of lordosis in response to estrogen, fail to exhibit an increase in muscarinic binding in the VMH following administration of estradiol [7, 14, 17]. Consequently, the feminization of behavior as determined by the prenatal hormonal environment may be correlated with the ability of estrogen to regulate certain neurochemical parameters in specific brain regions. In the present experiment, we attempted to determine if a prenatal treatment that feminizes the sexual behavior of male rats would also enhance the ability of estrogen to increase muscarinic binding in the MBH.

## METHOD

### *Subjects*

Long-Evans female rats (Charles River Company) were time-mated in our laboratory at approximately 100 days of age. On days 10–21 of gestation, pregnant females were injected intramuscularly with one of the following daily treatments: flutamide (5 mg in 0.1-ml volumes of propylene glycol, Schering Corporation) or propylene glycol (0.1 ml). Offspring were delivered naturally at 23 days of gestation. Males from mothers treated with flutamide or propylene glycol and females from mothers treated with propylene glycol were weaned at 21 days of age and housed in group cages by litter. Each treatment group consisted of animals from 5 litters.

At 90–100 days of age, all animals were gonadectomized. Several animals from each litter were selected randomly for biochemical analysis. The remaining male littermates from mothers treated with flutamide or propylene glycol were utilized for behavioral tests.

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*Biochemical Analysis*

Three weeks after gonadectomy, animals were injected intramuscularly for 3 days with 5 μg/kg estradiol benzoate (EB, Sigma Chemical Company) or 0.1 ml sesame seed oil vehicle. Approximately 24 hr after the final injection, animals were decapitated and the brains were removed rapidly. The MBH, medial preoptic area (POA), septum, and portions of the parietal cortex were dissected in ice-cold saline as illustrated in Fig. 1. Tissues were homogenized in 1 ml of ice-cold 320 mM sucrose. Homogenates were centrifuged at 1000 g for 10 min at 4°C and the supernatant was stored at −80°C for approximately 1 week prior to assay. Aliquots of supernatant (50 μl for MBH and septum, 100 μl for POA and cortex) were incubated for 45 min at 37°C in a medium containing 320 mM sucrose, 10 mM potassium phosphate, 1 mM EDTA, and 0.5 mM ascorbate (pH 7.4) with 0.5 nM tritiated 3-quinuclidinyl benzilate (QNB, 40 Ci/mmole, New England Nuclear). Since Scatchard analysis has revealed that the change in muscarinic binding induced by estrogen reflects a change in the number of binding sites [7, 14, 16, 17] only a single concentration of QNB was utilized in the present experiment. The final total incubation volume was 0.25 ml. Following incubation, bound ligand was separated from free ligand by filtration under vacuum through Whatman GF/B glass fiber filters. Filters were rinsed 4 times with 5 ml of ice-cold buffer, transferred to glass scintillation vials, and allowed to dry. Econofluor counting solution (10 ml, New England Nuclear) was added and samples were counted 24 hr later at approximately 45% efficiency. In order to determine the amount of non-specific binding, parallel incubations were run in buffer containing 0.5 nM tritiated QNB and 1 μM atropine sulfate, a competing muscarinic antagonist. Specific binding is defined as binding in the absence of atropine sulfate minus binding in the presence of atropine sulfate. Protein content of each sample was determined by the method of Bradford [1] with bovine-serum albumin standards. Values are expressed as fmol of QNB specifically bound per mg of protein.

*Behavioral Tests*

Four weeks after castration, male littermates from mothers treated with flutamide or propylene glycol were injected intramuscularly for three days with 4 μg EB. Approximately 24 hr after the final injection, males were tested for lordosis in a Plexiglas arena (45×50×58 cm) occupied by a stimulus male and the occurrence of lordosis, a concave arching of the spine, was recorded. The incidence of lordosis is expressed as a lordosis quotient: (number of lordoses/10 mounts)×100. This procedure was repeated the following week under the same hormonal regimen.

## RESULTS

Muscarinic binding in the four brain areas assayed is presented in Table 1. Estrogen treatment significantly increased muscarinic binding in the MBH of female rats treated prenatally with propylene glycol ($p<0.01$, $t$-test) but failed to alter binding in the MBH of control males treated prenatally with propylene glycol. However, estrogen did not increase muscarinic binding in the MBH of male rats that had been treated prenatally with flutamide. There were no changes induced by estrogen in any other brain areas of either sex.

Female sexual behavior exhibited by male littermates treated with propylene glycol or flutamide is presented in Table 2. Males exposed to flutamide prenatally displayed a significantly greater incidence of lordosis on two weekly tests than control males ($p<0.01$, $t$-test).

Finally, testicular descent failed to occur in 93% of all males treated prenatally with flutamide.

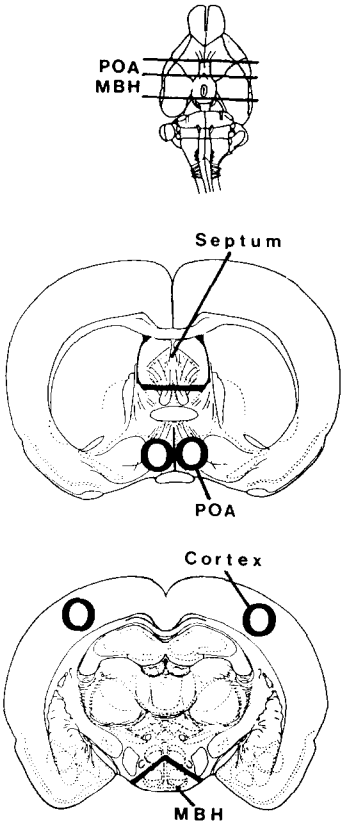


FIG. 1. Fresh brains were blocked from the ventral surface into two coronal segments corresponding to the medial preoptic area (POA) and the medial basal hypothalamus (MBH). Dissections were taken from these segments under a dissecting microscope with the aid of a capillary tube or scalpel. The coronal sections illustrated represent the approximate midpoint of the dissected regions. Adapted from Luine, Khylchevskaya, and McEwen [11] and Konig and Klippel [10].

## DISCUSSION

Male rats treated prenatally with the antiandrogen, flutamide, displayed an increased incidence of female sexual behavior as adults following treatment with estrogen. However, unlike normal female rats, males exposed to flutamide prenatally failed to exhibit an increased level of muscarinic binding in the MBH when treated with estrogen in adulthood. These results indicate that the behavior of male rats can be partially feminized without a corresponding change in the ability of estrogen to regulate muscarinic binding sites in the hypothalamus.

Several points regarding these results require elaboration. The differentiation of female sexual behavior in rats is believed to begin during the prenatal period and continue through early postnatal life [19]. This laboratory has shown previously that male rats treated prenatally with the antiandrogen, flutamide, display elevated levels of female

TABLE 1

EFFECT OF ESTRADIOL BENZOATE (EB) ON MUSCARINIC BINDING IN THE MEDIAL BASAL HYPOTHALAMUS (MBH), MEDIAL PREOPTIC AREA (POA), SEPTUM, AND PARIETAL CORTEX OF RATS TREATED PRENATALLY WITH PROPYLENE GLYCOL OR FLUTAMIDE

| | Propylene Glycol/Female | | Propylene Glycol/Male | | Flutamide/Male | |
|---|---|---|---|---|---|---|
| | Oil | EB | Oil | EB | Oil | EB |
| MBH | 324 ± 18 | 416 ± 25* | 347 ± 25 | 305 ± 28 | 330 ± 44 | 284 ± 12 |
| POA | 410 ± 35 | 407 ± 32 | 430 ± 37 | 426 ± 42 | 360 ± 49 | 349 ± 34 |
| Septum | 617 ± 82 | 598 ± 80 | 732 ± 37 | 816 ± 59 | 728 ± 24 | 663 ± 36 |
| Cortex | 1143 ± 157 | 1163 ± 104 | 1011 ± 67 | 1040 ± 118 | 941 ± 90 | 960 ± 48 |

EB (5 μg/kg) or sesame oil (0.1 ml) was administered for three days prior to sacrifice. Values represent mean fmol [$^3$H] QNB specifically bound per mg protein ± S.E.M. Each value is the mean of 8–9 determinations.

*$p<0.01$, vs. oil, $t$-test.

TABLE 2

FEMALE SEXUAL BEHAVIOR OF MALE RATS TREATED PRENATALLY WITH PROPYLENE GLYCOL OR FLUTAMIDE

| | Week 1 | Week 2 |
|---|---|---|
| Propylene Glycol (n=8) | 6 ± 3 | 16 ± 2 |
| Flutamide (n=8) | 38 ± 3* | 60 ± 3* |

Estradiol benzoate (4 μg) was administered for 3 days prior to testing on both weeks. Values represent mean lordosis quotients ± S.E.M.

*$p<0.01$, vs. propylene glycol, $t$-test.

sexual behavior as adults following estrogen treatment [9]. Assuming that an effective concentration of flutamide was not present in neonatal males immediately after birth, prenatal treatment with flutamide would not alter normal postnatal differentiation of the male brain. Consequently, the postnatal component of differentiation would not be affected by prenatal exposure to flutamide. It is possible, therefore, that the ability of the hypothalamus to respond to estrogen later in life is a result of early postnatal organization, rather than prenatal differentiation. Additional experiments employing neonatal castration of male rats would be required to fully address this hypothesis.

It is also worth noting that some investigators have found that changes in cholinergic neurochemistry induced by estrogen treatment are localized in discrete subregions of hypothalamus and basal forebrain as revealed by micropunch dissections [13, 16, 17]. The dissection procedure utilized in the present experiment sampled a wide locus of the hypothalamic region that included the anterior hypothalamus, ventromedial hypothalamus, arcuate nucleus, and probably portions of the dorsomedial and mammillary nuclei. Consequently, the size of the dissection might have masked small increases in muscarinic binding induced by estrogen in males treated prenatally with flutamide.

Central cholinergic activity has a clear and reliable effect on the display of female sexual behavior in the rat [2, 3, 4, 6]. Estrogen, the primary hormone regulating this behavior, is capable of altering a number of cholinergic endpoints in the forebrain including muscarinic receptor binding [7, 8, 14, 16, 17] and cholinergic enzyme activity [12,13]. However, the anatomical link between estrogen regulation of this system and control of female sexual behavior has yet to be identified. It is possible that a change in hypothalamic muscarinic binding induced by estrogen is not a critical factor in the mediation of female sexual behavior in rats. Recently, we have obtained evidence that the MBH may not be the principle site at which elevations in cholinergic activity act to facilitate lordosis behavior in female rats [5]. In addition, although estrogen has been reported to alter muscarinic binding in the POA of female rats [7, 14, 17] this area also does not appear to be a principle cholinergic locus that regulates lordosis behavior [5]. The possibility remains that estrogen-induced changes in muscarinic binding in brain areas other than the MBH and POA may influence female behavior.

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