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Sex Differences in Progesterone Receptor Expression: A Potential Mechanism for Estradiol-Mediated Sexual Differentiation

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The differential exposure of males and females to testosterone (T) and its metabolite estradiol (E) contributes to the development of sex differences in the brain. However, the mechanisms by which T and E permanently alter neural development remain virtually unknown. Two regions of the rat preoptic area, the anteroventral periventricular nucleus (AVPv) and the medial preoptic nucleus (MPN), are sexually dimorphic and serve as models for studying the hormonal mechanisms of sexual differentiation. Around birth, these regions express dramatically higher levels of progesterone receptor immunoreactivity (PRir) in males than they do in females. The present study examined the possibility that sexually dimorphic induction of PR expression in these two regions constitutes a potential mechanism of E-mediated sexual differentiation. Prenatal exposure to either T propionate or the synthetic estrogen, diethylstilbestrol, but not dihydrotestosterone propionate, significantly increased PRir levels in the MPN and AVPv of fetal females compared with controls. Prenatal exposure to the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione, significantly reduced PRir in the MPN and AVPv of fetal males, whereas the androgen receptor antagonist flutamide had no effect. This suggests that aromatization of T into E is crucial for the sex difference in PR expression in the MPN and AVPv during development. (*Endocrinology* 143: 3727–3739, 2002)

STEROID HORMONES AND their receptors are powerful regulators of gene transcription in the nervous system and have the potential to permanently alter the structure and function of the developing brain. Sexual differentiation, the process by which permanent sex differences in the brain arise, is regulated by testosterone (T) secreted from the fetal and neonatal testes. There are two surges in circulating T during early development to which many of the reported sex differences in the brain have been attributed, one occurring sometime between embryonic d 18 and 19 (E18 and E19), and another occurring just a few hours after birth (1, 2). Within the brain, T can be metabolized to estradiol (E) by the enzyme aromatase, and it is this conversion that is responsible for producing many of the sex differences in the brain.

Two regions of the rat preoptic area are especially sensitive to the effects of T and its metabolite, E, on development, the anteroventral periventricular nucleus (AVPv) and the medial preoptic nucleus (MPN). These areas are sexually dimorphic in their structure, neurochemistry, and function (3–9). For example, the volume of AVPv (6, 8) and a subnucleus of the MPN (the MPNc; Refs. 3 and 10) are sexually dimorphic, as are several neuropeptidergic and neurotransmitter systems in these two regions (Refs. 4 and 5; for review see Ref. 11). Additionally, the MPN mediates sexually dimorphic behaviors (12, 13), whereas the AVPv regulates gonadotropin secretion, the pattern of which is sexually dimorphic (14, 15). Many of the sex differences in anatomy and function of the AVPv and MPN are the result of differential exposure of males and females to T and subsequently E, during perinatal development (8, 12, 16–19).

While the importance of T and E in sexual differentiation is not in question, the cellular and molecular mechanisms by which these steroids sexually differentiate the AVPv and MPN are not well understood. In addition, the question of whether T, acting through E, is solely responsible for sexual differentiation of these two regions remains unanswered.

During late prenatal and early postnatal periods of development, male rats have much higher levels of progesterone receptor (PR)-immunoreactivity (PRir) than females in the MPN (20) and the AVPv (Ref. 21; and present paper), suggesting that these regions may be differentially sensitive to progesterone in males and females during the developmental period critical for sexual differentiation. We offer the possibility that the sexually dimorphic induction of PR expression mediates the effects of T on sexual differentiation of the brain.

Several lines of evidence are consistent with the idea that gonadal steroid hormones are responsible for PR induction in the fetal and neonatal brain. Before E18, PR expression in the MPN and AVPv is undetectable in males and females. However, beginning on E19/E20, PRir becomes evident in males, producing a significant sex difference (Ref. 20; and our unpublished observations). The induction of PRir in the

Abbreviations: ATD, 1,4,6-Androstatriene-3,17-dione; AVPv, anteroventral periventricular nucleus; DES, diethylstilbestrol; DHTP, dihydrotestosterone propionate; E, estradiol; E18 or E19, embryonic d 18 or 19; ER, estrogen receptor; MPN, medial preoptic nucleus; MPNc, subnucleus of the MPN; NGS, normal goat serum; PG, propylene glycol; PR, progesterone receptor; PRir, progesterone receptor immunoreactivity; T, testosterone; TBS, Tris-buffered saline; TP, T propionate.

MPN and AVPv of males corresponds precisely with the time of the established prenatal surge in circulating T (1). Furthermore, the preoptic area of the male fetus expresses the enzymes aromatase and 5α-reductase (22–24), indicating that the fetal brain has the capacity to locally convert T to its active metabolites, E and dihydrotestosterone (24–27). This suggests that T or its metabolites could be responsible for the induction of PR expression in the fetal male preoptic area.

The dramatic sex difference in PRir persists postnatally until approximately postnatal d 10, at which time females begin to express PR in the MPN and AVPv, and the magnitude of the sex difference is reduced (our unpublished observations). The induction of PRir in the postnatal female MPN and AVPv roughly coincides with the onset of ovarian steroidogenesis (Refs. 28–31; and our unpublished observations), suggesting that ovarian hormones may be inducing PR expression in the female MPN and AVPv around postnatal d 10.

The temporal correspondence of these hormonal events and the differential expression of PR in males and females during this developmental period suggest that gonadal steroid hormones are most likely responsible for PR induction in the MPN and AVPv of the male fetus. The present study examined the hypothesis that T and/or one of its metabolites regulate PR expression in these regions leading to the sex difference in the expression of this receptor. The results show that T and its estrogenic, but not its androgenic metabolites, induced PRir in the developing AVPv and MPN. Therefore, regulation of the PR gene may be one mechanism by which E masculinizes the brain.

Materials and Methods

General procedures

Animals. Adult female Sprague Dawley rats (60–80 d of age; Taconic Laboratories, Germantown, NY) were housed with males of the same strain on a 10-h light, 14-h dark cycle at a constant temperature of 25 ± 2 C. The day copulatory plugs were found was designated d 1 of gestation (E1). Pregnant females were singly housed in plastic tubs with bedding and given food and water *ad libitum*. All animal procedures used in these experiments were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst.

Tissue preparation and sex determination. Because hormone administration might delay or otherwise interfere with normal parturition, fetuses were delivered by Caesarian section on the morning of E22 for experiments 2–5. Pregnant females were given a lethal dose of pentobarbital:chloral hydrate (0.25 M chloral hydrate, 0.08 M magnesium sulfate, 45 mM pentobarbital, 3 M ethyl alcohol, 4.5 M propylene glycol in distilled water), the abdominal cavity was opened, and the uterine horns were removed and placed on ice. Fetuses were removed, anesthetized on ice, and killed by decapitation. Brains from fetuses and neonates were removed from the skull and immediately immersion-fixed in 5% acrolein in 0.1 M phosphate buffer (pH 7.6) for 6 h, then cryoprotected in 30% sucrose in 0.1 M phosphate buffer until the time of sectioning. The anogenital distance (*i.e.* the distance from the tip of the phallus to the end of the anal opening) of each fetus from experiments 4 and 5 was measured using microcalipers and a dissecting microscope. The sex of each fetus was determined by the presence of testes in males and uterine horns in females with the aid of a dissecting microscope.

All brains were sectioned on a freezing microtome in the coronal plane at 50 μm. Sections were stored in cryoprotectant (30% sucrose, 0.1% polyvinyl-pyrrolidone-40 in ethylene glycol, and 0.1 M phosphate buffer) at −20 C until immunocytochemical processing.

Immunocytochemistry. Immunocytochemistry was performed on free-floating sections using a rabbit polyclonal antiserum (DAKO Corp. Inc., Glostrup, Denmark) directed against the DNA binding domain of the human PR. This antibody detects both the A and B isoforms of PR (32). All incubations were performed at room temperature unless otherwise indicated. Sections were rinsed in Tris-buffered saline (TBS; pH 7.6) three times for 5 min to remove any residual cryoprotectant solution. Sections were then incubated in 1% sodium borohydride in TBS for 10 min, rinsed in TBS four times for 5 min each, and then incubated in TBS containing 20% normal goat serum (NGS), 1% H_2O_2 and 1% BSA for 30 min. With the exception of experiment 3, in which a dilution of 1:500 was used, PR antiserum was diluted to 1:1000 in TBS containing 2% NGS, 0.3% Triton X-100, and 0.02% sodium azide for 72 h at 4 C. Following three rinses (5 min each) in TBS containing 2% NGS, 0.3% Triton X-100, and 0.02% sodium azide, the sections were incubated for 60 min in biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) at a concentration of 5 μg/ml in TBS containing 2% NGS, 0.3% Triton X-100, and 0.02% sodium azide. After two rinses (5 min each) in TBS containing 2% NGS, 0.3% Triton X-100, and 0.02% sodium azide and two rinses (5 min each) in TBS, the sections were incubated in avidin-biotin complex reagent (Vectastain Elite Kit, Vector Laboratories) for 60 min. Following three rinses (5 min each) in TBS, the sections were incubated in TBS containing 0.05% diaminobenzidine, 0.75 mM nickel ammonium sulfate, 0.15% β- D-glucose, 0.04% ammonium chloride, and 0.001% glucose oxidase for approximately 20 min. The sections were then rinsed three times (5 min each) in TBS and mounted on gelatin-coated slides and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Immunocytochemical controls. The production and specificity for the polyclonal PR antisera that was raised against human PR has been described previously (32). In the present study, PR immunocytochemistry was performed on E22 male AVPv and MPN sections as described above. However, the following permutations were conducted to test for antibody specificity. Some sections were incubated in either 1:1000 or 1:500 DAKO Corp. PR antisera that had been preabsorbed overnight at 4C with either 200 μg/ml or 300 μg/ml of the antigen peptide, respectively (Ref. 32; amino acids 533–547; Genosys Biotechnologies, Inc., The Woodlands, TX). Additional sections were incubated in 1:1000 DAKO Corp. PR antisera that had been preabsorbed with a 10-fold molar excess of human PR (A and B isoforms). Human PR-A and PR-B proteins were obtained from Sf9 insect cells infected with the appropriate PR recombinant transfer plasmids as described previously (Ref. 33; Tissue Culture CORE Facility, University of Colorado Cancer Center, Denver, CO). Yet other AVPv and MPN sections were incubated with untreated PR antisera (DAKO Corp.) as a positive control or were incubated in buffer alone, with the primary antisera omitted. MPN and AVPv sections incubated in preabsorbed antiserum or incubated in the absence of primary antibody had a complete absence of nuclear immunoreactivity compared with positive controls (Fig. 1).

Analysis. For all experiments, a representative section through the rostral MPN and AVPv of each subject was selected for image analysis by an experimenter blind to treatment group. Each section of the MPN and the AVPv were anatomically matched across animals using distinguishing landmarks (34). Animals from which an anatomical match could not be found due to damaged sections were excluded from analysis. Microscopic images of the PRir in the MPN and AVPv were captured with an Olympus Corp. BH-2 microscope fitted with a CCD72 (Dage MTI, Michigan City, MI) camera that was connected to a QuickCapture frame grabber board (Data Translation Inc., Marlboro, MA) in a MacIntosh IIfx computer. NIH Image software (W. Rasband, National Institutes of Health, Bethesda, MD) was used to analyze captured images. The relative amount of PRir in the MPN and AVPv was determined by measuring the area (μm^2) covered by "thresholded" pixels [*i.e.* those pixels with a gray level higher than a defined threshold density (specific immunoreactive staining)]. "Threshold" was defined as the mean optical density three to five times (depending on the experiment) the SD higher than the mean background density. The mean background density was measured in a region devoid of PRir, immediately lateral to the analyzed region containing PRir. For experiments 2, 4, and 5, statistical analyses were performed using two-way ANOVA ($P < 0.05$), followed by pairwise comparisons using Student-Newman-Keuls *post hoc* analysis ($P < 0.05$). For experiment 1, statistical analyses were performed using Student's *t* test ($P < 0.05$), whereas for experiment 3, statistical analyses comparing females across hormonal manipulations were performed

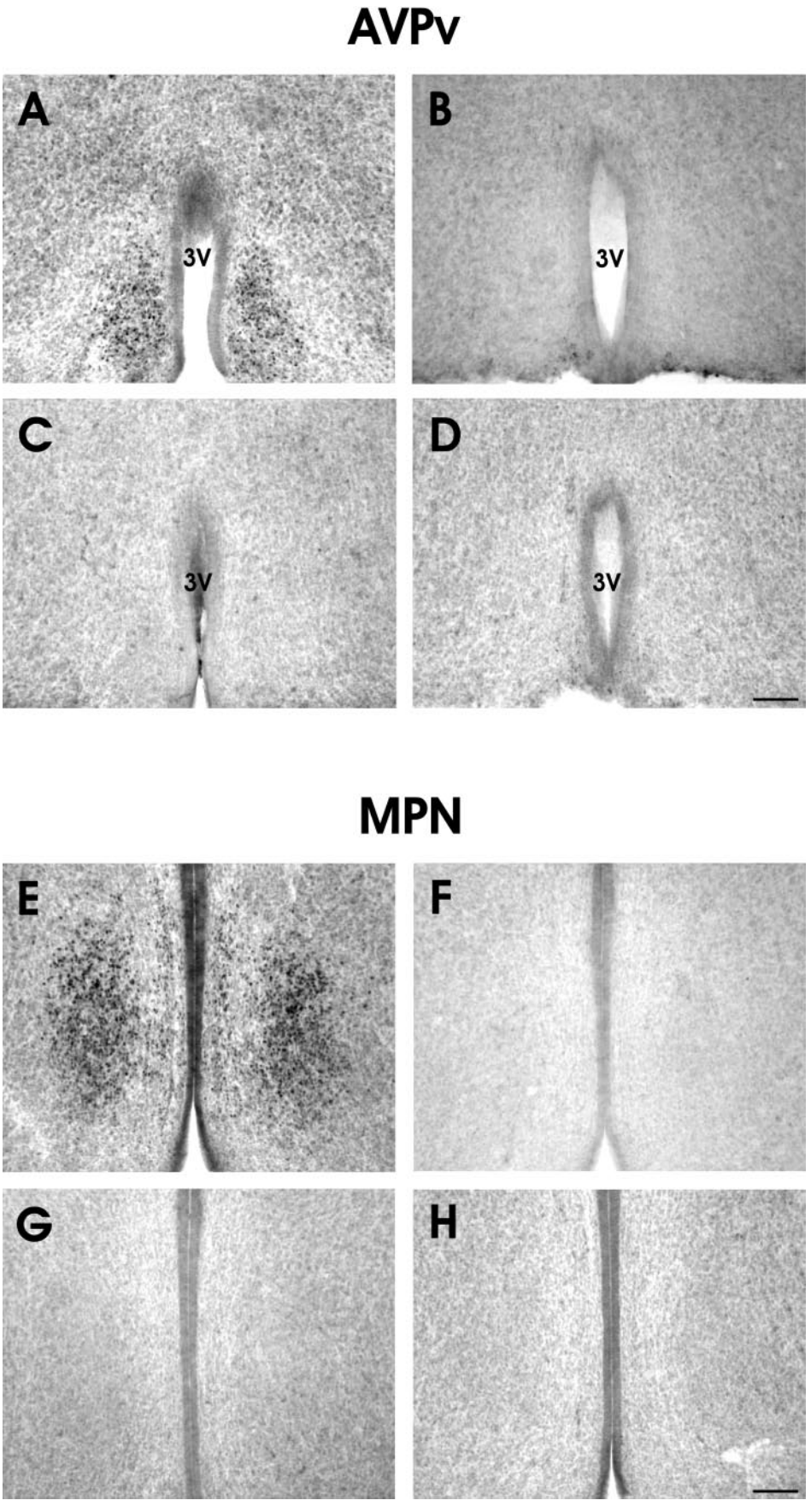

FIG. 1. Immunocytochemical controls for PR polyclonal antisera. Representative coronal sections of the AVPv (A–D) and the MPN (E–H) of E22 males were incubated with (A and E) untreated PR antisera (1:1000); B and F, buffer alone with PR antisera omitted; C and G, PR antisera preabsorbed with human PR (A and B isoforms); or D and H, PR antisera preabsorbed with 200 μg/ml of the peptide antigen. 3V, Third ventricle. *Bar*, 100 μm.

using one-way ANOVA ($P < 0.05$), followed by pairwise comparisons using Student-Newman-Keuls *post hoc* analysis.

Experimental design

Experiment 1: sex difference in PR in the AVPv. Five adult females were mated as described above, left undisturbed for the duration of their pregnancy, and allowed to undergo normal parturition on E23. Within 24 h following parturition, one or two males and one or two females from each litter (males n = 9; females n = 9) were killed by decapitation, following anesthesia on ice. Brains were processed for PR immunocytochemistry and analyzed as described above.

Experiment 2: effect of prenatal exposure to T. Ten pregnant females received daily sc injections of T propionate (TP; Sigma, St. Louis, MO) (2 mg/0.1 cc sesame oil; n = 5 pregnant females) or received an equal volume of the oil vehicle (n = 5 pregnant females) from E16–E21. This dose and timing of TP administration masculinizes the neuromuscular system, the spinal nucleus of the bulbocavernosus (35), another sexual dimorphism of the CNS that develops prenatally. On E22, fetal brains representing all five litters in each hormonal condition (n = 8–10 subjects of each sex per group), were collected and processed for immunocytochemistry and analyzed as described above.

Experiment 3: effect of prenatal exposure to T and its metabolites. Twelve pregnant females received daily sc injections of either TP (2 mg; n = 3 pregnant females), the synthetic estrogen, diethylstilbestrol (DES, Sigma) (20 μg; n = 3 pregnant females), dihydrotestosterone propionate (DHTP, Sigma) (2 mg; n = 3 pregnant females) in 0.1 cc sesame oil or an equal volume of the oil vehicle alone (n = 3 pregnant females) from E19–E22. Because circulating levels of α-fetoprotein may decrease the potency of E in fetal females (36), the synthetic estrogen DES was used in place of E in this experiment. DES has a much lower affinity for α-fetoprotein (37), and this prenatal dose has previously been used to masculinize the MPN of females (38). Hormone administration was delayed until E19 in experiment 3 because pilot studies revealed that administration of DES to pregnant females on E17 resulted in spontaneous abortion of the fetuses by E19. All pregnant females, receiving DES from E19–E22 in the present study, maintained pregnancy normally until the time the rats were killed, on E22. Female fetal brains representing all three litters in each hormonal condition (each group contained 4 or 5 fetal subjects of each sex per group), were processed for immunocytochemistry and analyzed as described above.

Experiment 4: effect of prenatal exposure to an aromatase inhibitor. Ten pregnant females received sc injections of either the aromatase inhibitor, ATD [1,4,6-androstatriene-3,17-dione, Steraloids, Inc. (Newport, RI); 5 mg/0.05 cc propylene glycol (PG)], or a similar volume of PG vehicle (5 pregnant females per treatment) from the evening of E16 until the evening of E21. This dose and timing of ATD has been previously shown to alter the sexual differentiation of MPN morphology as well as components of male sexual behavior (39), a behavior mediated by the MPN (12). On E22, fetal brains representing all five litters from each hormonal treatment (n = 9 or 10 fetal subjects of each sex per group) were collected and processed for immunocytochemistry and analyzed as described above. Previous studies have suggested that ATD can potentially bind to the androgen receptor *in vitro* (40, 41). Although the dose of ATD used in the present study has been shown to demasculinize brain function and behavior, anogenital distance was measured in all fetuses as a bioassay for any general androgenic effect of ATD. Anogenital distance was chosen as the bioassay because it is sexually dimorphic and the sex difference is almost exclusively androgen dependent (42, 43).

Experiment 5: effect of prenatal exposure to an androgen receptor antagonist. Eleven pregnant females received daily sc injections of the androgen receptor antagonist flutamide (5 mg/0.05 cc PG; Sigma; n = 6 pregnant females), or a similar volume of PG (n = 5 pregnant females) from the evening of E16 until E21. This prenatal dose of flutamide significantly demasculinizes the androgen-dependent, sexually dimorphic system of the spinal nucleus of the bulbocavernosus (44). The anogenital distance of each fetus was also assessed to ensure that our dose of flutamide was sufficient to demasculinize the fetal male. On E22, fetal brains representing all litters from each hormonal group (n = 10–12 fetal subjects of each sex per group) were processed for immunocytochemistry and analyzed as described above.

Results

Experiment 1: PR expression on the day of birth

A significant sex difference in PRir was observed in the AVPv on the day of birth. Males had significantly higher levels of PRir compared with females ($P < 0.0001$) (Fig. 2). A similar sex difference in PRir in the MPN has been reported previously (20).

Experiment 2: prenatal exposure to T

AVPv. Prenatal exposure to TP significantly increased PRir levels in the AVPv of E22 females (Figs. 3A and 4, A–D). Two-way ANOVA revealed a significant main effect of sex, males had higher levels of PRir than females ($F_{(1, 31)} = 12.31$, $P < 0.005$), and a significant sex X hormone treatment interaction ($F_{(1, 31)} = 39.34$, $P < 0.0001$). *Post hoc* analysis revealed that control males and TP-treated females had significantly more PRir in the AVPv compared with control females and TP-treated males ($P < 0.05$). Additionally, PRir levels in TP-treated females were significantly lower than control males ($P < 0.05$).

MPN. Prenatal exposure to TP significantly increased PRir levels in the MPN of E22 females and abolished the sex difference in PR expression (Figs. 3B and 4, E–H). Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 35)} = 30.79$, $P < 0.0001$) and a significant sex X hormone treatment interaction ($F_{(1, 35)} = 50.89$, $P < 0.0001$). *Post hoc* analysis revealed that control males and TP-treated females had significantly higher levels of PRir in the MPN than control females ($P < 0.05$). Additionally, control males had significantly higher levels of PRir than TP-treated males and females ($P < 0.05$).

Experiment 3: prenatal exposure to T metabolites

AVPv. Prenatal exposure to TP and DES but not DHTP significantly increased PRir in the AVPv of E22 females (Fig. 5A). One-way ANOVA revealed a significant effect of treatment ($F_{(3, 13)} = 16.20$, $P < 0.0001$). *Post hoc* comparisons revealed that both TP-treated and DES-treated females had higher levels of PRir compared with oil-treated females ($P < 0.05$). Furthermore, DES-treated females had significantly higher levels of PRir than TP-treated females ($P < 0.05$). There were no significant differences between oil-treated and DHTP-exposed females.

MPN. Exposure to prenatal TP and DES, but not DHTP, significantly increased PRir in the MPN of E22 females (Fig. 5B). One-way ANOVA revealed a significant effect of treatment ($F_{(3, 15)} = 21.4$, $P < 0.0001$). *Post hoc* comparisons indicate that TP-treated females and DES-treated females had higher levels of PRir compared with oil- and DHTP-treated females ($P < 0.05$). No significant differences were observed between DES- and TP-treated females, nor between DHTP- and oil-treated females.

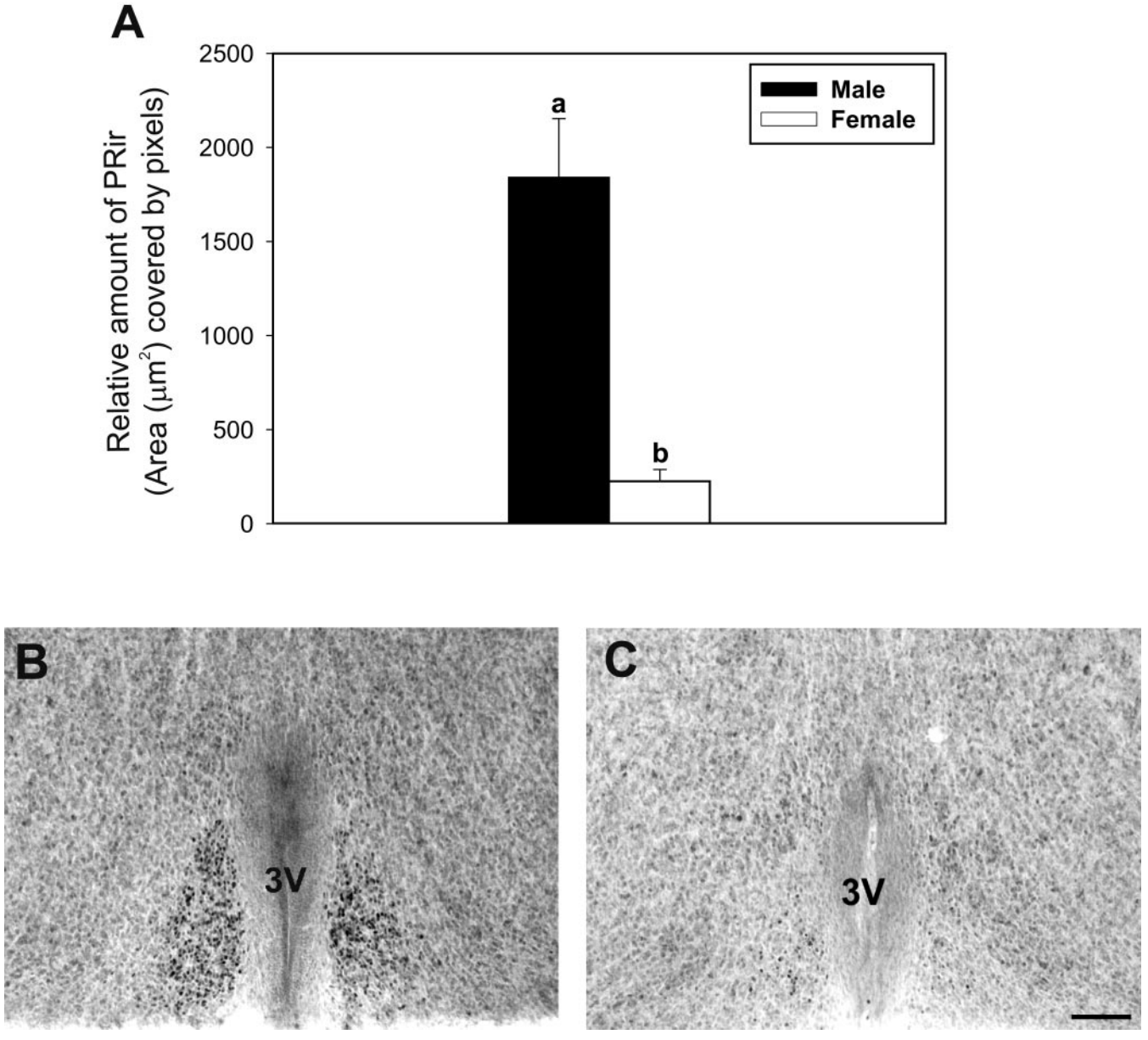

FIG. 2. Sex difference in PR expression in the AVPv. A, Relative levels of PRir in males (*black bar*) and females (*white bar*) on the day of birth in the AVPv. *Bars with different letters* are statistically different from each other, $P < 0.0001$. Representative coronal section from the AVPv of (B) a male and (C) a female on the day of birth. 3V, Third ventricle. *Bar*, 100 μm. Males, n = 9; females, n = 9.

Experiment 4: effect of prenatal exposure to the aromatase inhibitor ATD

Anogenital distance. The mean anogenital distance for males and females from each litter was determined and statistical analyses were performed on these values. Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 16)} = 1485.51$, $P < 0.0001$) but no effect of treatment. There was no significant interaction. *Post hoc* comparisons revealed that both control and ATD-treated males had greater anogenital distances compared with their female counterparts ($P < 0.05$). See Table 1 for specific mean (±SEM) values for each group.

AVPv. Prenatal exposure to the aromatase inhibitor ATD reduced levels of PRir in the AVPv of E22 males (Fig. 6A). Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 33)} = 40.10$, $P < 0.0001$) and a main effect of treatment ($F_{(1, 33)} = 41.6$, $P < 0.0001$), as well as a significant sex X treatment interaction ($F_{(1, 33)} = 37.2$, $P < 0.0001$). *Post hoc* analyses revealed that control males had significantly higher levels of PRir in the AVPv than control females ($P < 0.05$). Control males also had significantly higher levels of PRir compared with ATD-treated males ($P < 0.05$). No significant differences were found between ATD-treated males and females or ATD-treated females and control females.

MPN. Prenatal administration of the aromatase inhibitor, ATD, significantly reduced levels of PRir in the MPN of E22 males, thereby abolishing the sex difference (Figs. 6B and 8,

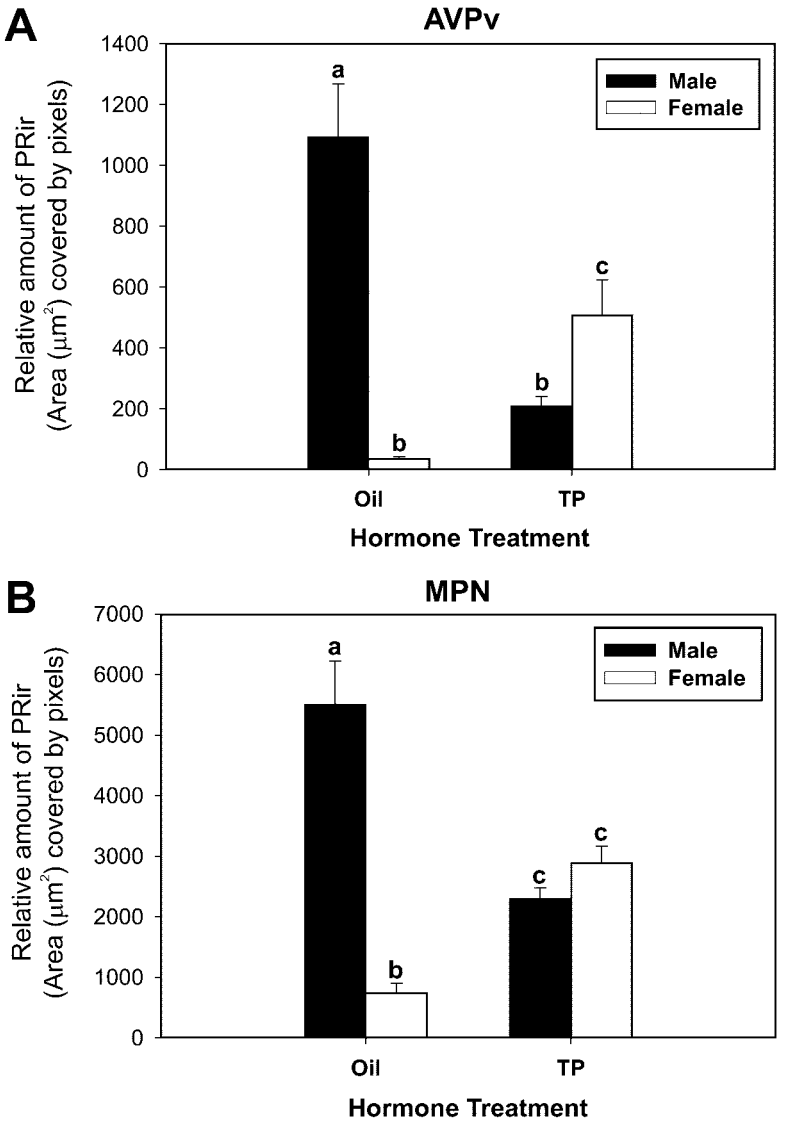

FIG. 3. Prenatal T exposure induces PR expression in the AVPv and the MPN. Relative levels of PRir in the (A) AVPv and (B) MPN of E22 males (*black bars*) and females (*white bars*), treated with 2 mg of TP or an equal volume of oil vehicle from E16–E21. *Bars with different letters* are statistically different from each other ($P < 0.05$). AVPv: Oil males, n = 9; oil females, n = 9; TP males, n = 8; TP females, n = 9. MPN: Oil males, n = 9; oil females, n = 10; TP males, n = 10; TP females, n = 10.

A and B). Two-way ANOVA revealed a significant main effect of sex $F_{(1, 35)} = 134.0$, $P < 0.0001$) and main effect of treatment ($F_{(1, 35)} = 138.4$, $P < 0.0001$). Additionally, there was a significant sex X treatment interaction ($F_{(1, 35)} = 119.0$, $P < 0.0001$). *Post hoc* analysis revealed that control males had higher levels of PRir than control females ($P < 0.05$). PRir in ATD-treated males was decreased compared with control males ($P < 0.05$). Additionally, ATD-treated males did not differ in their levels of PRir from control or ATD-treated females. There was no significant effect of treatment in females.

Experiment 5: prenatal exposure to androgen receptor antagonist

Anogenital distance. Exposure to flutamide significantly demasculinized anogenital distance. Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 18)} = 658.8$, $P < 0.0001$), and a significant main effect of treatment ($F_{(1, 18)} = 404.9$, $P < 0.0001$), as well as a significant sex X treatment interaction ($F_{(1, 18)} = 406.6$, $P < 0.0001$). *Post hoc* comparisons indicated that control males had significantly larger anogenital distances than control females and flutamide-treated males ($P < 0.05$). Furthermore, flutamide-treated males had significantly larger anogenital distances compared with flutamide-treated and control females ($P < 0.05$). There were no significant differences between control and flutamide-treated females. See Table 1 for specific mean (±SEM) values for each group.

AVPv. Exposure to flutamide did not significantly alter the levels of PRir in the AVPv on E22 (Fig. 7A). Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 36)} = 113.79$, $P < 0.0001$), but no effect of treatment or a significant interaction. *Post hoc* comparisons indicated that control and flutamide-treated males had significantly higher levels of PRir in the AVPv compared with respective female counterparts ($P < 0.05$).

MPN. Exposure to flutamide did not significantly alter the levels of PRir in the MPN on E22 (Figs. 7B and 8, C and D). Two-way ANOVA revealed a significant main effect of sex ($F_{(1, 38)} = 230.27$, $P < 0.0001$). There was no significant main

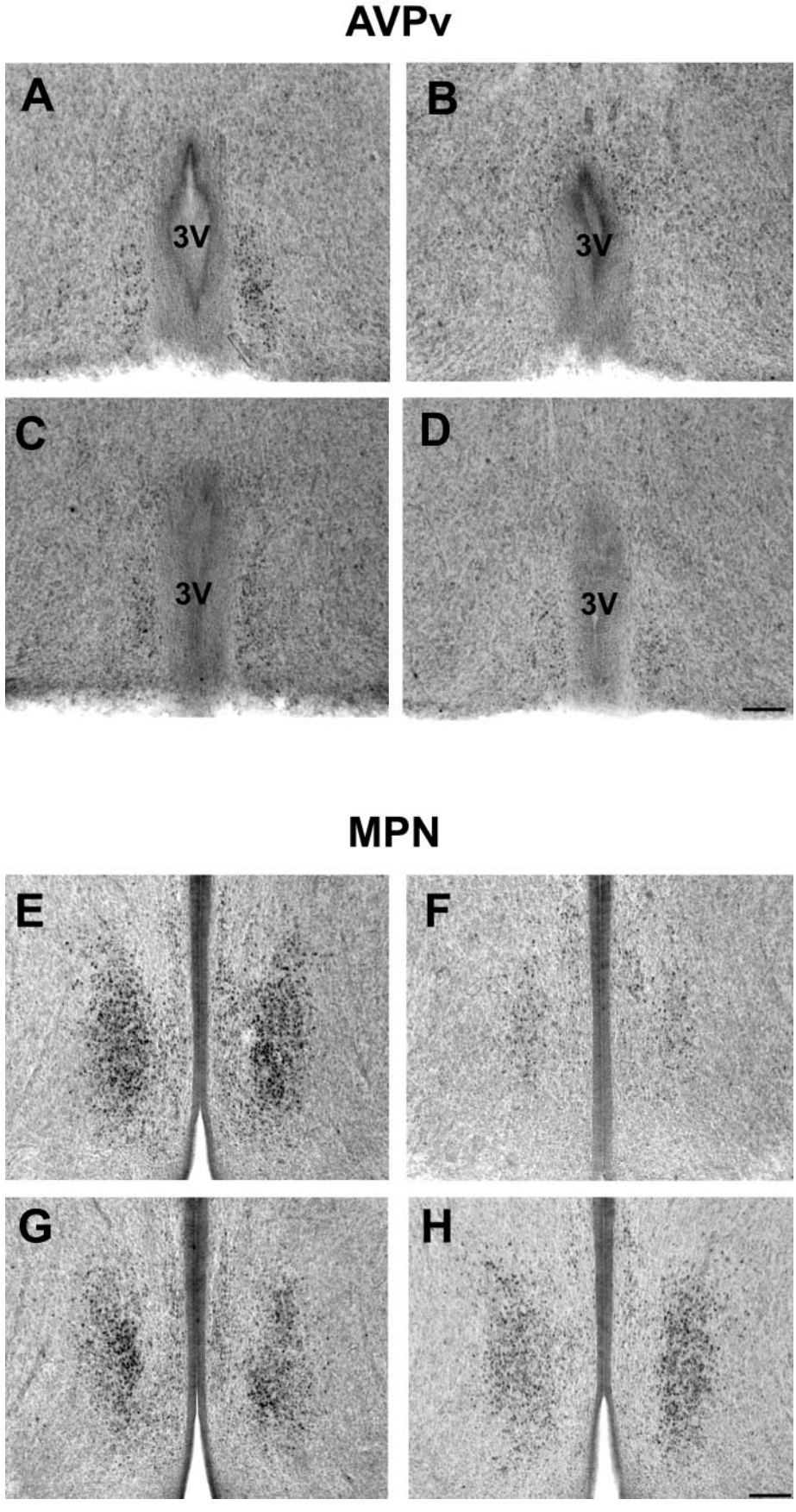

FIG. 4. Representative coronal sections from the (A–D) AVPv and (E–H) MPN of E22 fetuses from each treatment group. A and E, Oil-treated male; B and F, oil-treated female; C and G, TP-treated male; D and H, TP-treated female. 3V, Third ventricle. *Bar*, 100 μm.

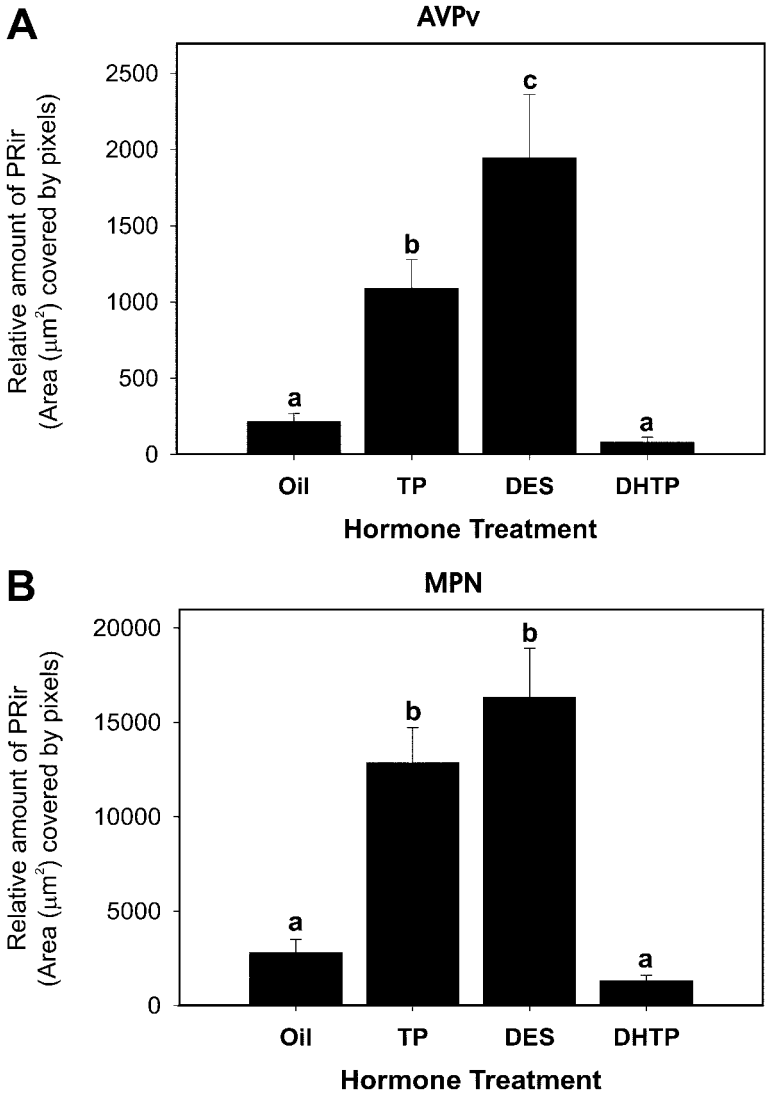

FIG. 5. Induction of PRs in the preoptic nuclei by estrogenic, and not androgenic, metabolites of T. Relative levels of PRir in (A) the AVPv and (B) the MPN of E22 females that were treated with either TP, DES, DHTP, or sesame oil from E19–E22. *Bars with different letters* are statistically different from each other ($P < 0.05$). AVPv: Oil females, n = 4; TP females, n = 4; DHTP females, n = 5; DES females, n = 4. MPN: Oil females, n = 5; TP females, n = 4; DHTP females, n = 5; DES females, n = 5.

TABLE 1. Mean (±SEM) anogenital distances (mm) of E22 males and females from experiments 4 and 5

Treatment	Male	Female
A) Experiment 4: aromatase inhibitor		
Propylene glycol	2.91 ± 0.03	1.29 ± 0.03
ATD	2.95 ± 0.03	1.33 ± 0.03
B) Experiment 5: androgen receptor antagonist		
Propylene glycol	3.10 ± 0.06	1.19 ± 0.04
Flutamide	1.42 ± 0.04	1.19 ± 0.03

effect of treatment. *Post hoc* comparisons revealed that both control and flutamide-treated males had significantly higher levels of PRir in the MPN than control and flutamide-treated females ($P < 0.05$).

Discussion

This study investigated the hormonal regulation of the sexually dimorphic expression of PR in two preoptic nuclei, specifically the AVPv and the MPN. Similar to what has been previously reported for the MPN (20), there is a dramatic sex difference in the expression of PR in the AVPv on the day of birth, where males have higher levels of PRir than do females. The regulation of PR expression by steroid hormones in these two preoptic nuclei is quite similar. In general, T and its estrogenic metabolite, E, appear to be responsible for the induction of PR in the fetal male AVPv and MPN. Prenatal exposure to T and the synthetic estrogen, DES, induced PR expression in the two preoptic nuclei of females, whereas prenatal treatment with an aromatase inhibitor reduced PR expression in the AVPv and MPN of males. In contrast, prenatal treatment with either the androgenic metabolite of T, DHTP, or the androgen receptor antagonist, flutamide, did not significantly alter PR expression in the AVPv or MPN of either sex. These results suggest that the induction of PR by E may be one mechanism by which T sexually differentiates the AVPv and MPN.

Although this study is the first to document the hormonal regulation of PR expression in the fetal brain, the present results are consistent with previous work examining the regulation of PR in the adult and neonatal brain. E replacement in ovariectomized females restored PR mRNA, protein, and binding to levels seen in controls (45–48). In a similar

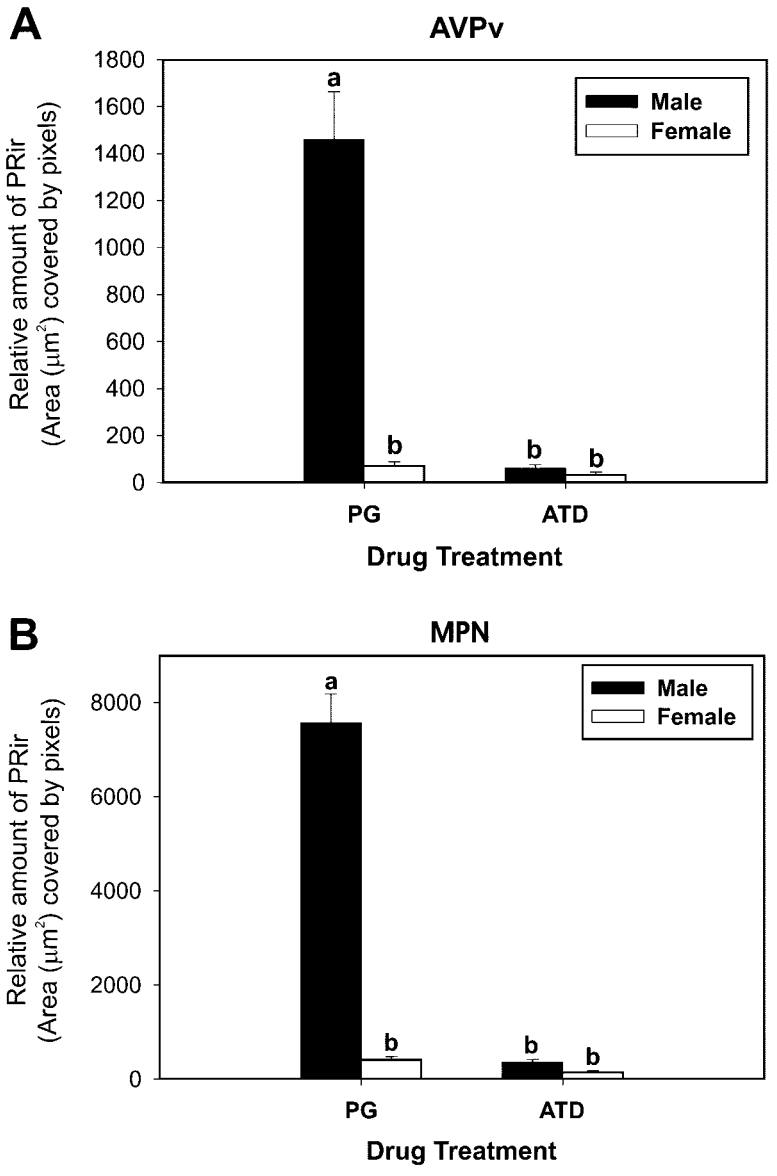

FIG. 6. Reduction in PR expression in two preoptic nuclei with prenatal aromatase inhibitor treatment. Relative levels of PRir in (A) the AVPv and (B) the MPN of E22 males (*black bars*) and females (*white bars*) that were treated with 5 mg of the aromatase inhibitor ATD or PG (vehicle) from E16–E21. *Bars with different letters* are statistically different from each other ($P < 0.05$). AVPv: PG males, n = 10; PG females, n = 9; ATD males, n = 9; ATD females, n = 9. MPN: PG males, n = 10; PG females, n = 10; ATD males, n = 10; ATD females, n = 9.

manner, DES or E administration increased PR binding in the hypothalamus/preoptic area in the postnatal female (49–51). These findings, together with the present study, support the idea that PR expression in the preoptic area, throughout life, depends on estrogenic stimulation.

Nuclear estrogen receptors (ERs) are the primary means by which E exerts its effects in the brain. Currently, there are two known forms of the ER (ERα and ERβ; Refs. 52–54). ERα is the receptor through which E acts to induce PR expression in the adult brain (55, 56) and is expressed at high levels in the fetal and neonatal preoptic area (57–59). Additionally, there are several consensus sequences for estrogen response elements in the promoter region of the PR gene, and site-directed mutagenesis of these regions results in a blunted E-induced expression of PR (60). In fact, ERα is necessary for the sexually dimorphic induction of PR in several regions of the developing brain, including the AVPv (our unpublished observations) and the MPN (61). Developing mice, similar to rats, express PR in a sexually dimorphic manner in these regions and neonatal male mice that lack a functional gene for ERα (ERαKO) express dramatically lower levels of PR compared with wild-type counterparts (Ref. 61; and our unpublished observations). This suggests that during development, the PR gene is directly regulated by E's action on ERα, resulting in a dramatic sex difference in PR expression during a period that is critical for sexual differentiation of the brain.

While E appears to be the most potent regulator of PR gene expression, it appears that androgens may also influence the expression of these receptors. PRir was significantly reduced in the AVPv and MPN of males that had been treated prenatally with T (experiment 2). Consistent with the present study, the androgenic metabolite of T, DHT, reduced PR expression in T47D human breast cancer cells (62). The effects of androgens in the current study may reflect a direct action of androgen receptors on the PR gene. On the other hand, androgens may regulate PR indirectly through a down-regulation of the ER gene (63).

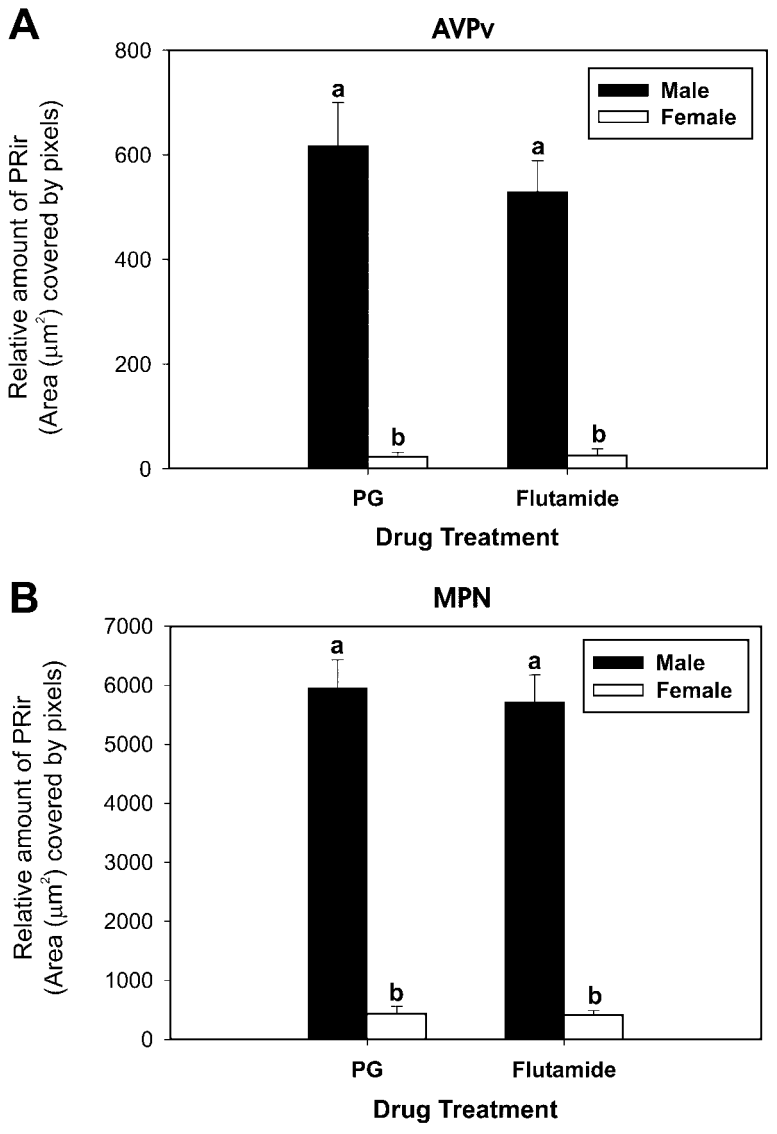

FIG. 7. Effect of exposure to prenatal androgen receptor antagonist flutamide on PR expression in two preoptic nuclei. Relative levels of PRir in (A) the AVPv and (B) the MPN of E22 males (*black bars*) and females (*white bars*) that were exposed to 5 mg of flutamide or PG (vehicle) from E16–E21. *Bars with different letters* are statistically different from each other ($P < 0.05$). AVPv: PG males, n = 10; PG females, n = 10; flutamide males, n = 10; flutamide females, n = 10. MPN: PG males, n = 10; PG females, n = 10; flutamide males, n = 12; flutamide females, n = 10.

Although the AVPv and MPN are sexually differentiated by gonadal hormones, T has opposing effects in these two areas. For example, the most striking sex difference in the MPN is in the volume of its subnucleus, the MPNc, which is 3–7 times larger in males than in females (3, 10). However, the volume of the AVPv, is larger in females than in males (6–8). Interestingly, these opposing structural dimorphisms can both be attributed to the differential exposure of males and females to T during critical periods of development (8, 16). In both these regions, T exposure influences programmed cell death or apoptosis, a mechanism that contributes to the morphological sex differences in both preoptic nuclei, but it does so in opposite directions. More specifically, T increases the incidence of cell death in the AVPv but decreases it in the MPN (10, 64). This suggests that although T is the common factor in the sexual differentiation of the AVPv and MPN, it may activate divergent mechanisms leading to the development of opposing sex differences in these two regions.

There is evidence to support the idea that PR may be the point of divergence in T's regulation of apoptosis. There exist as many reports of progesterone preventing apoptosis in some tissues as there are reports of progesterone inducing apoptosis in other tissues. Previous studies demonstrate both PR-dependent up-regulation and down-regulation of anti-apoptotic gene expression (65–67). In contrast, the breast cancer literature (65, 68, 69) and the one study examining regulation of apoptotic genes in neural tissue by E, the aromatized metabolite of T (70) are remarkably consistent; they all demonstrate an up-regulation of pro-life proteins with estrogen treatment. Therefore, the evidence points to progesterone, and not E, as potentially having a dual role in the process of apoptosis. In effect, progesterone may be capable of inducing cell death in some populations of cells (*e.g.* AVPv), while promoting cell survival in others (*e.g.* MPN). This suggests that PR induction in the AVPv and MPN could be a mechanism through which T can have differential effects on the molecular machinery regulating apoptosis.

Recent evidence from our lab has demonstrated a potential role for PR in the sexual differentiation of the MPN structure

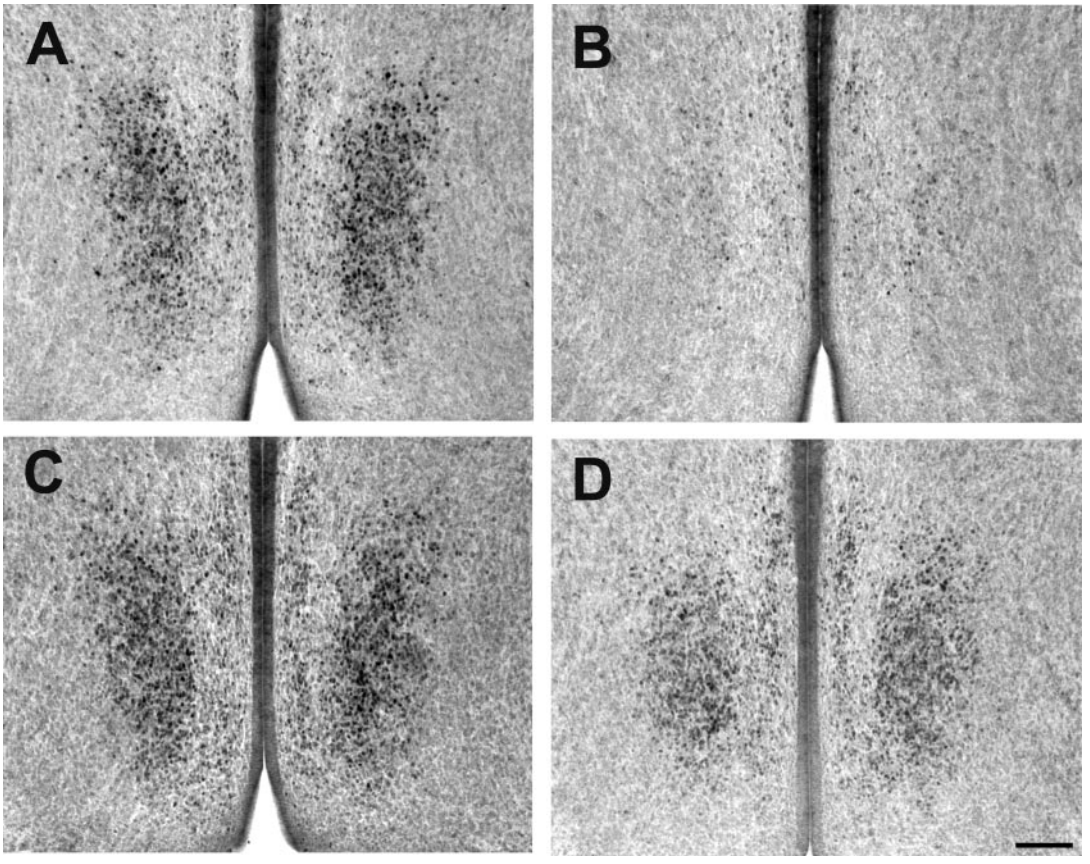

FIG. 8. Representative coronal sections from the MPN of E22 males treated with (A) PG; (B) ATD; (C) PG; and (D) flutamide. PRir is shown here as *black dots* on either side of the third ventricle. *Bar*, 100 μm.

and function. As previously mentioned, the MPNc volume of adult males is significantly larger than females (3, 10), a direct result of exposure to T during perinatal life (16, 10). Blocking PR function during the first week of life with the PR antagonist, RU486, attenuated the effects of T in masculinizing the MPNc volume in females (71). Functionally, the MPN mediates sexually dimorphic male copulatory behavior (12) and neonatal RU486 treatment abolished subsequent adult sexual behavior in most males (72). In addition, others have demonstrated that adult male sexual behavior was reduced in mice lacking a functional progesterone receptor (PRKO) gene (73). Findings from these studies suggests that PR plays a role in the development of the morphological and behavioral sex differences of the MPN and implicates progesterone as a potential factor in the process of sexual differentiation.

Although the source of ligand for PR perinatally is presently unknown, there are at least two possible sources of progesterone. Circulating progesterone levels do not differ between males and females *in utero* (1, 74, 75), suggesting that the source of progesterone is not the fetal ovary. However, circulating levels of progesterone are positively correlated between fetuses and their mothers during the last few days of pregnancy (74, 75), suggesting that the primary source of progesterone in fetal circulation is the mother. Additionally, we have demonstrated that progesterone from maternal circulation can enter fetal circulation and bind to PR in the fetal male MPN (75), making the mother a potential source of ligand for PR in the fetal brain. Another possible source of ligand may be the *de novo* synthesis of progesterone within the developing brain itself because the neonatal rat brain appears to express the enzymes necessary for progesterone synthesis (76, 77). The sex difference in PR expression suggests that the developing preoptic area of males is more sensitive to progesterone. Therefore, regardless of the source of ligand, progesterone may have differential effects on the brains of the two sexes.

T and its metabolite, E, are responsible for generating many of the neuroanatomical, neurochemical, and functional sex differences in the brain. The molecular cascade activated by T and E exposure during development remains poorly understood. However, results from the present study indicate that a direct effect of E exposure during development is the induction of PR expression in the preoptic area. Thus, PR may be the next step in the cascade of T-mediated sexual differentiation.

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