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Unexpected Effects of Perinatal Gonadal Hormone Manipulations on Sexual Differentiation of the Extrahypothalamic Arginine-Vasopressin System in Prairie Voles

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The sexually dimorphic extrahypothalamic arginine-vasopressin (AVP) projections from the bed nucleus of the stria terminalis to the lateral septum (LS) and lateral habenula (LHb) are denser in males than females and, in rats, require males' perinatal exposure to gonadal hormones but the absence of such exposure in females. We examined perinatal hormone effects on development of this sex difference in prairie voles (Microtus ochrogaster), which show atypical effects of hormones on sexual differentiation of some reproductive behaviors. Neonatal castration reduced the number of AVP mRNA-expressing cells in the bed nucleus of the stria terminalis and AVP immunoreactivity (ir) in the LS and LHb. Surprisingly, daily injections of 1000 µg of testosterone propionate (TP) during the first postnatal week did not maintain high levels of AVP-ir in neonatally castrated males. Furthermore, perinatal treatments with TP (75, 500, or 1000 µg), testosterone (100 µg), or dihydrotestosterone (200 µg) did not masculinize AVP-ir in the female LS or LHb. In fact, 1000 µg TP reduced it in some cases. However, 1000 µg TP lengthened anogenital distance, indicating that TP was biologically active. Neonatal estrogen receptor antagonism with tamoxifen reduced AVP-ir in the male LS, whereas treating neonatal females with the synthetic estrogen diethylstilbestrol increased septal AVP-ir. Tamoxifen and diethylstilbestrol had no effects in the LHb. Similar to rats, therefore, postnatal estrogen influences some components of the extrahypothalamic AVP system in prairie voles, but this developing system appears to be insensitive to exogenous androgens, including aromatizable androgens. Such insensitivity is atypical for a sexually dimorphic neural system in a rodent and may reflect the unusual effects of hormones on sexual differentiation of some behaviors in prairie voles. (Endocrinology 146: 1559–1567, 2005)

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 Abbreviations: ATD, 1,4,6-Androstatriene-3,17-dione; AVP, arginine-vasopressin; BST, bed nucleus of the stria terminalis; DES, diethylstilbestrol; DHT, dihydrotestosterone; ir, immunoreactivity; LHb, lateral habenula; LS, lateral septum; MA, medial amygdala; PB, phosphate buffer; PVT, paraventricular thalamic nucleus; TAM, tamoxifen; TBS, Tris-buffered saline; TP, testosterone propionate.

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male prairie voles, but perinatal treatment of virgin females with TP does not masculinize it (25, 26). The influence of gonadal hormones on the development of any sex difference in the prairie vole brain has not been examined and may differ from rats. The present studies investigated perinatal hormone effects on the development of sexually dimorphic AVP expression.

Materials and Methods

Subjects

Subjects were F4 and F5 generation prairie voles (M. ochrogaster) that were born and raised in our colonies, which were established in 1996 at the University of Massachusetts, Amherst, and in 2002 at Michigan State University, from breeding stock originating from offspring of voles captured in 1994 from Urbana, IL, provided by Dr. Betty McGuire (Smith College, Northampton, MA) and Dr. Zuoxin Wang (Florida State University, Tallahassee, FL). These stocks were outbred most recently in 2000, with voles of Illinois origin provided by Dr. C. Sue Carter (University of Illinois at Chicago, Chicago, IL). Animals were mated by socially isolating adult virgin female and male prairie voles for 3 d, after which females were placed in the cage of an unfamiliar male. Animals were maintained on a 14-h light, 10-h dark cycle with an ambient temperature of 21°C. At all ages, animals were housed in plastic cages (48 x 28 x 16 cm) containing wood chips, wood shavings, and a substantial hay covering. Water and a food mixture containing cracked corn, whole oats, sunflower seeds, and Purina rabbit chow (ratio of 1:1:2.2) were available ad libitum.

In experiments in which pups were manipulated prenatally via injections given to their dam (experiments 2 and 4), litters were fostered on the day of birth to unmanipulated surrogate lactating prairie voles from our colony that had given birth to a litter within the previous 24 h. Similar to our previous work (25, 26), animals were housed in single-sex groups of two to four animals per cage. Subjects remained housed with their same-sex littermates until killed at approximately 90 d old. Body weight and anogenital distance were measured either at the time of birth to unmanipulated surrogate lactating prairie voles (27) and fixed 2 wk later. Sections were then lightly counterstained with methyl green, dried, and coverslipped.

Analysis of AVP fibers and cells

All slides were randomly coded and for analysis. Density of AVP-ir fibers in the LS, LHB, and, in experiment 1, the paraventricular thalamic

through the heart with 50 ml of 0.9% saline followed by 50 ml of 5% acrolein in sodium phosphate buffer (PB). Brains were postfixed overnight in 5% acrolein in PB and then submerged in a 30% sucrose-PB solution for 3 d until sectioning. Subjects whose brains were used for in situ hybridization analysis were rendered unconscious with carbon di- oxide and decapitated. Brains were rapidly removed from the skull, flash-frozen in cold 2-methyl butane, and stored at –70°C until sectioning.

In situ hybridization

Brains were cut into 40-μm sections with a freezing microtome, and immunocytochemistry for AVP was performed on every third section throughout the entire brain as described previously (29). Sections were rinsed between each step three times for 5 min in Tris-buffered saline (TBS), incubated in 1% sodium borohydride for 10 min, 1% Triton X-100 and 1% hydrogen peroxide in TBS for 10 min, 20% normal goat serum in 0.3% Triton X-100 in TBS for 10 min, and rabbit anti-AVP primary antibody (1:15,000; ICN Biochemicals, Aurora, OH) at room temperature for approximately 18 h. Sections were then rinsed three times with 0.3% Triton X-100 in TBS, incubated in a biotinylated goat antirabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA), rinsed three times in TBS for 5 min each, and incubated with avidin-biotin complex (Vectastain Elite; Vector Laboratories) for 60 min. Visualization of AVP immunoreactivity (ir) occurred with a solution of 0.05% 3’-3’-diaminobenzene tetrahydrochloride, 0.04% ammonium chloride, 0.0004% glucose oxidase, 0.75 mg nickel ammonium sulfate, and 0.3% β-D-glucose. Sections were mounted on microscope slides, dehydrated, and coverslipped.

Killing

Subjects in all experiments were killed 3 wk after implantation of T-filled capsules. Subjects whose brains were used for immunocytochemistry were overdosed with ketamine and xylazine and perfused with 2% sodium chloride (SSC)-0.25% acetic anhydride (pH 8.0). Slides were then dehydrated and coverslipped.

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Adult gonadectomy and testosterone (T) capsule implantation

Perinatal treatment of pups is described in detail for each experiment later in this article. In all cases, when pups reached 70–75 d age, they were anesthetized with an ip injection of ketamine (62.5 mg/kg), and acepromazine (0.8 mg/kg), and gonadally intact males and females were gonadectomized. Neonatally castrated males received a sham surgery. All subjects then received a single 2.5-μm ISLASTIC brand capsule (Dow Corning, Midland, MI) filled with crystalline T implanted sc in the nape of their neck. Circulating levels of T in adult voles after implantation of a 2.5-μm long capsule have not been determined but are likely supraphysiological. The size of this capsule is consistent with the only other studies on the extrahypothalamic AVP system in prairie voles and is capable of maintaining normal extrahypothalamic AVP expression in adult males (27).

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nucleus (PVT) was examined in sections of randomized and coded slides as described previously (29) with computerized image analysis using the NIH IMAGE 1.44 software (National Institutes of Health, Bethesda, MD). Fiber density was analyzed by gray-level thresholding and was expressed as the total area (in pixels) within a sample region covered by AVP-ir fibers. Measurements were standardized across brain sections by keeping the mean OD of the background constant. AVP-ir fiber density in the LS and LHb was measured bilaterally in an area of 450 × 350 μm from two sections per site per subject that had the highest fiber density; three sections per subject were analyzed bilaterally in the PVT as a control site in experiment 1. Within the LS, the area analyzed included the ventrolateral LS approximately at the level immediately rostral to the decussation of the anterior commissure, an area which covers the densest part of the AVP fiber plexus in this region and corresponds approximately to plate 18 of Paxinos and Watson’s atlas of the rat brain (30). In the LHb, the area of analysis included a caudal LHb region that has the densest AVP fiber plexus, corresponding approximately to plate 33 of Paxinos and Watson (30). Sections chosen for analysis of the PVT included the same sections that were analyzed for the LHb plus the next section lying immediately caudal.

The total number of AVP mRNA-expressing cells in six hemisections through the BST and MA were quantified by eye under bright-field illumination by a single observer (J.S.L.) unaware of subject condition. Grain density was determined by computerized gray-level thresholding in the four hemisections that contained the most AVP mRNA-expressing cells and analyzed with NIH IMAGE 1.44. From these four hemisections, 10 cells within the BST of each subject were randomly chosen for analysis, along with five cells from the MA. Grain density was expressed as the number of pixels that each cell had covered by grains.

Data analyses

Immunocytochemical data from experiments 1, 3, and 5–7 were analyzed with one-way ANOVA followed by Bonferroni post hoc tests. In situ hybridization data from experiment 2 were also analyzed with a one-way ANOVA followed by Bonferroni post hoc tests. Computerized image analysis data from experiment 4 were analyzed with a one-way ANOVA with control males included and then a two-way ANOVA including data only from females using prenatal and postnatal treatments as factors, with both of the ANOVAs followed by Bonferroni analysis. Data from males in experiment 8 were analyzed with an unpaired t test; data from females in experiment 8 failed the assumption of homogeneity of variance and were instead analyzed with a Mann-Whitney U test. Statistical significance was indicated by P ≤ 0.05.

Results

Experiment 1: effects of neonatal castration on AVP-ir

Methods. Neonatal males were cryoanesthetized on ice within 2–12 h after birth. One group of males (n = 11) received two lateral incisions of their ventrum, and their testes were removed. The other half of the males received a sham castration (n = 8). A group of control neonatal females (n = 11) were also cryoanesthetized and received a sham surgery. Incisions were closed with silk suture. Pups were placed under a warm lamp until they were active, after which, they were returned to their dam. When 70–75 d old, subjects were gonadectomized (or received a sham surgery), implanted with a T-filled SILASTIC brand capsule, and killed 3 wk later as detailed above. Brains were later processed for AVP immunocytochemistry as detailed above.

Results. One neonatal sham male from experiment 1 was found to be an outlier in the amount of AVP-ir in the LS (Dixon’s outlier test, P ≤ 0.01) and was removed from the analysis for that site. Control males had significantly more AVP-ir in the LS than control females, and neonatally castrated males had levels of AVP-ir in the LS that were intermediate between the control males and females (F(2,27) = 9.19, P ≤ 0.001), with all groups significantly different from each other, (P ≤ 0.05; Figs. 1 and 2). A similar pattern of differences between groups was also found in the LHb (F(2,28) = 7.71, P ≤ 0.003; Figs. 1 and 2).

Experiment 2: effects of perinatal hormone manipulations on AVP mRNA expression in males

Methods. Pregnant females (n = 13) received a daily sc injection of 5 mg of the androgen receptor blocker flutamide (a,α,α-trifluoro-2-methyl-4-nitro-m-propionotoluidide; Sigma, St. Louis, MO) dissolved in 100 μl sesame oil immediately followed by a sc injection of 1 mg of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD; Steraloids Inc., Wilton, NH) dissolved in 100 μl sesame oil beginning 1 wk after pairing with a male. To generate control subjects, other pregnant females (n = 13) received two injections of 100 μl of sesame oil each day. The gestation period in prairie voles is similar to rats, so prenatal treatments were intended to cover at least the last 2 wk of the 3-wk gestation period. Our previous studies show that parturition occurs approximately 15 d after the beginning of the dams’ treatment with either ATD, flutamide, or sesame oil (25, 26). Doses were chosen based on the effectiveness of similar or even smaller doses to influence sexual differentiation in rats (for references, see Ref. 28). Within 12 h after birth, half of the males from each group were castrated, and the other half received a sham surgery as described earlier. All pups from all groups were fostered to recently parturient surrogate lactating dams from our colony. When 70–75 d old, all subjects were gonadectomized (or received a sham surgery if neonatally castrated), implanted with a T-filled capsule, and killed 3 wk later. Brains were processed for in situ hybridization as described earlier. The sample sizes for the male groups were as follows: prenatal oil/neonatal sham, n = 8; prenatal oil/neonatal castration, n = 7; prenatal ATD + flutamide/neonatal sham, n = 7; and prenatal ATD + flutamide/neonatal castration, n = 8. A group of neonatal females that was cryoanesthetized and received a sham surgery (n = 6), followed by ovariectomy and T capsule implant during adulthood, was also included in the study.

Results. We were unable to collect data from the BST of one female, both the BST and MA of one sham male, and the BST of one neonatally castrated male. Another neonatally cas-

FIG. 1. AVP-ir (mean ± SEM pixels covered by immunoreactive fibers) in the LS and LHb of female prairie voles and male prairie voles that received a neonatal sham surgery or neonatal castration. Different letters above bars indicate significant differences between groups within each brain region; P ≤ 0.05.
A trated male was found to be an outlier in the number of AVP mRNA-expressing cells in the BST (Dixon’s outlier test, \( P \leq 0.01 \)) and was removed from data analyses for this site. There were no significant effects of prenatal treatment with flutamide plus ATD on any measure, and groups of males were, therefore, collapsed across prenatal treatment for further analysis.

There were significant differences between groups in the number of AVP mRNA-expressing cells in the BST (\( F(2,32) = 5.85, P = 0.007 \); Figs. 3 and 4). Sham-castrated males had the greatest number of AVP mRNA-expressing cells, which was significantly greater than both neonatally castrated males and control females, which were statistically similar to each other. There were no significant differences between groups in the number of grains per cell within the BST (\( F(2,32) = 1.50, P \geq 0.2 \); data not shown).

Within the MA, there were no significant differences between any of the groups in the number of AVP mRNA-expressing cells (\( F(2,34) = 0.18, P \geq 0.8 \); Fig. 3) or in the number of grains per cell (\( F(2,34) = 1.41, P \geq 0.2 \); data not shown).

**Experiment 3: effects of T replacement on AVP-ir in neonatally castrated males**

**Methods.** Neonatal males were cryoanesthetized and received either a sham surgery or neonatal castration. Immediately after surgery and continuing once a day for the next 6 d, sham subjects (\( n = 8 \)) received a sc injection of 100 \( \mu l \) sesame oil, and the neonatally castrated subjects received a daily injection of 100 \( \mu l \) sesame oil (\( n = 8 \)) or 1 mg TP in 100 \( \mu l \) oil (\( n = 13 \)). Subjects were raised to adulthood, received a sham surgery, or were castrated as detailed above, and then implanted with a SILASTIC brand capsule filled with T and killed 3 wk later. Brains were later processed for AVP immunocytochemistry.

**Results.** Similar to experiment 1, neonatal castration significantly reduced AVP-ir in the LS (\( F(2,26) = 16.33, P \leq 0.0001 \)). However, treating neonatally castrated males with TP during the first week of life after neonatal castration did not maintain high levels of AVP-ir but, instead, nonsignificantly decreased AVP-ir (Fig. 5). A similar effect was found in the LHb but the difference between the sham control males and neonatally castrated males that had received oil only approached statistical significance (\( P = 0.053 \)). Neonatally castrated males that received oil or TP did not have a general deficiency in synthesizing AVP because they did not differ from each other or sham control males in the level of AVP-ir in the PVT (\( F(2,26) = 0.19, P \geq 0.82 \); data not shown).

**Experiment 4: effects of prenatal and/or postnatal TP treatment on AVP-ir in females**

**Methods.** Pregnant female prairie voles received either a daily injection of 50 \( \mu g \) TP dissolved in 100 \( \mu l \) sesame oil (\( n = 16 \) dams) or 100 \( \mu l \) sesame oil (\( n = 16 \) dams) beginning 1 wk after pairing with a male until the birth of pups. As noted above, this procedure encompassed the last 2 wk of the 3-wk gestation period, as shown in our previous studies (25, 26). Beginning on the day of birth and continuing for the next 6 d, half of the litter from each prenatal treatment group was injected sc each day with 1 mg TP in 50 \( \mu l \) sesame oil. The other half was injected each day with 50 \( \mu l \) sesame oil. When they were adults, females were ovariectomized, received a T-filled capsule, and killed 3 wk later. The final sample sizes were as follows: prenatal oil/postnatal oil, \( n = 9 \); prenatal oil/postnatal TP, \( n = 7 \); prenatal TP/postnatal oil, \( n = 8 \); and prenatal TP/postnatal TP, \( n = 11 \). A group of males (\( n = 9 \)) exposed to sesame oil pre- and postnatally were gonadectomized as adults, implanted with a SILASTIC brand capsule filled with T, and killed 3 wk later were the positive control.

**Results.** All groups of females, regardless of perinatal treatment, had significantly less AVP-ir in the LS (\( F(4,39) = 14.46, P \leq 0.0001 \); Fig. 6) and LHb (\( F(4,39) = 12.849, P \leq 0.0001 \); Fig. 6) than control males. Two-way ANOVA on data from the LS of the female groups revealed no effect of prenatal TP (\( F(1,1) = 0.002, P \geq 0.96 \)) but a significant decrease in AVP-ir due to postnatal TP (\( F(1,1) = 5.41, P \leq 0.03 \)). There was no significant prenatal by postnatal interaction effect in the LS (\( F(1,1) = 0.06, P \geq 0.81 \)). Similar to the LS, females that received postnatal TP showed a significant decrease in AVP-ir in the LHb (\( F(1,1) = 9.72, P \leq 0.005 \)), and no effects of prenatal TP (\( F(1,1) = 0.34, P \geq 0.56 \)) or prenatal by postnatal interaction (\( F(1,1) = 0.01, P \geq 0.9 \)) were found.

*Fig. 2. Photomicrograph of AVP-ir in the LS of a representative control female (A), neonatally castrated male (B), and sham-operated control male (C) (right). LV, Lateral ventricle.

*Fig. 3. Number (mean \( \pm \) SEM) of AVP mRNA-expressing cells in the BST and MA of female prairie voles and male prairie voles that received either a neonatal sham surgery or neonatal castration. Different letters above bars indicate significant difference between groups within each brain region; \( P \leq 0.05 \).*
Experiment 5: effects of a single postnatal TP administration on AVP-ir in females

Methods. Females were injected once on postnatal d 3 with either 500 μg TP diluted in 50 μl sesame oil (n = 8) or sesame oil (n = 8). A group of males injected on postnatal d 3 with 50 μl sesame oil (n = 6) was used as the positive control. Subjects were gonadectomized as adults, received a T-filled capsule, and killed 3 wk later. Brains were later processed for AVP immunocytochemistry.

Results. A single injection of 500 μg TP on postnatal d 3, which has been reported to masculinize sexual behavior in female prairie voles (24), did not masculinize female AVP-ir to the level found in control males. Levels in both female groups were significantly lower than oil-treated control males in the LS (F(2,19) = 32.83, P = 0.0001) and LHb (F(2,19) = 13.024, P = 0.0003; Fig. 7). However, when just the female groups were analyzed with unpaired t tests, AVP-ir was greater in TP-treated females than oil-treated females in both the LS (t14 = 2.36, P = 0.04) and LHb (t14 = 2.24, P = 0.05).

Experiment 6: effects of repeated, but spaced, neonatal administration of TP on AVP-ir in females

Methods. Females were injected every third day from postnatal d 2–11 (i.e. on d 2, 5, 8, and 11) with either 500 μg TP diluted in 50 μl sesame oil (n = 9) or sesame oil (n = 8). A group of males injected on postnatal d 2, 5, 8, and 11 with 50 μl sesame oil (n = 7) was used as the positive control. Subjects were gonadectomized as adults, received a T-filled capsule, and killed 3 wk later. Brains were later processed for AVP immunocytochemistry.

Results. Extending TP treatment past the first neonatal week had no effect on AVP-ir in females, with both groups of females having significantly lower AVP-ir than oil-treated males in the LS (F(2,21) = 9.31, P = 0.002) and LHb (F(2,21) = 6.54, P = 0.05; Fig. 7).
significantly greater AVP-ir in the LS (U = 0.061; Fig. 10). Females treated neonatally with DES had 0.50, P = 0.003, and TAM also tended to reduce AVP-ir in the LHb (t18 = 0.19, P = 0.8). Adult body weight of males was not affected by any perinatal treatment in any experiment (all P ≥ 0.1; data not shown).

Also similar to results found previously (26), prenatal and postnatal treatment with TP significantly increased anogenital distance in the subset of females examined from experiment 4, although anogenital distance of both TP-treated and oil-treated females was shorter compared with control males (F(2,19) = 100.1, P = 0.0001; data not shown). In experiment polynomial regression between the levels of AVP-ir in the LS of DES-treated females and the sex ratio of their litter at birth (F(2,10) = 4.98, P = 0.04), with DES being more effective in masculinizing septal AVP-ir in females originating from litters that had more males.

Body weight and anogenital distance
As found previously (26), neonatal castration significantly reduced adult anogenital distance in males in experiments 1–3, and TP treatment increased it to control values in neonatally castrated males in experiment 3 (all P ≤ 0.01; data not shown). Treatment with TAM had no effect on male anogenital distance (t18 = 0.19, P = 0.8). Adult body weight of males was not affected by any perinatal treatment in any experiment (all P ≥ 0.1; data not shown).

Results. Males treated with TAM has significantly less AVP-ir in the LS than oil-treated males (t18 = 3.47, P ≤ 0.003), and TAM also tended to reduce AVP-ir in the LHb (t18 = 2.00, P = 0.061; Fig. 10). Females treated neonatally with DES had significantly greater AVP-ir in the LS (U = 12.50, P ≤ 0.001), but DES did not significantly affect AVP-ir in the LHb (t21 = 0.50, P = 0.6; Fig. 10). In addition, there was a significant
5. Injection of 500 μg TP once on postnatal d 3 did not affect anogenital distance, and the anogenital distance for both groups of females was shorter than males \( (F_{(2,19)} = 77.97, P \leq 0.0001) \). In Experiment 6, injection of 500 μg TP on postnatal d 2, 5, 8, and 11 also did not affect anogenital distance, and the distance was shorter for both groups of females than males \( (F_{(2,19)} = 56.64, P \leq 0.0001) \). In experiment 7, 1 mg TP lengthened female anogenital distance compared with other groups of females, whereas 75 μg TP, 100 μg T, and 200 μg DHT did not; anogenital distance in all groups of females was shorter than males \( (F_{(5,45)} = 41.92, P \leq 0.0001) \). Visual inspection of females from experiment 7 at death, however, revealed that all androgen-treated females had hypertrophied clitorises. DES treatment in experiment 8 had no effect on female anogenital distance \( (t_{21} = 1.0, P \geq 0.3) \). Adult body weight of females was not affected by any perinatal treatment in any experiment \( (P \geq 0.5) \); data not shown.

**Discussion**

Although some sex differences in the mammalian nervous system can be influenced by the social environment or sex differences in circulating gonadal hormones during adulthood \( (31–34) \), the magnitude of most identified sex differences in the rodent nervous system can be reduced or almost eliminated by manipulating gonadal hormone exposure during perinatal life. Indeed, the brains of male rats that are sheltered from perinatal androgenic or estrogenic activity through pharmacological agents prenatally and/or castration neonatally are demasculinized in many ways, including neuroendocrine release \( (35) \), structural morphology \( (36–40) \), hormone receptor expression \( (41–43) \), and neurotransmitter content \( (44–47) \). Conversely, most of these studies have demonstrated that treatment of perinatal females with androgens or estrogens significantly masculinizes these neural characteristics.

The same is true for the extrahypothalamic AVP pathways in rats. Castration of neonatal males \( (\text{before postnatal d 7}) \) completely demasculinizes AVP mRNA expression in the BST, as well as AVP-ir fiber density in the LS \( (12, 14, 48) \), whereas a single or repeated injection of TP to neonatally castrated males within the first 3 wk of life maintains masculinization of AVP mRNA expression in the BST and partially maintains masculinization of AVP-ir fibers in the LS \( (12, 13, 48) \). In females, injection of TP relatively soon after birth completely masculinizes AVP-ir fiber density in the LS \( (48) \), and either a single or repeated injection of TP or estradiol benzoate during this time masculinizes the number of AVP mRNA-expressing cells in the BST \( (12, 14) \).

The present results demonstrate that neonatal castration of male prairie voles produced effects similar to those in rats, that is, completely demasculinizing AVP mRNA expression in the BST and partially demasculinizing AVP-ir fiber density in the LS and LHb. The surprising finding was that, unlike rats, perinatal treatment with exogenous TP could not maintain masculine AVP expression in neonatally castrated males and did not masculinize this system in females. A similar lack of effects was found after neonatal treatment of females with T or DHT. The 1-mg dose of TP did increase the voles’ anogenital distance, as we have reported previously \( (26) \), indicating that TP is biologically active in this species. As far as we are aware, this is the first example of a demasculinization of the male rodent brain after neonatal castration without a restoration after exogenous androgen treatment, as well the first demonstration that perinatal androgens are ineffective in masculinizing a sexually dimorphic neural trait in a female rodent. Interestingly, the same effect can be found for sex differences in the parental behavior of virgin prairie voles, such that males are demasculinized by neonatal castration but the behavior of females is not masculinized by perinatal TP \( (26) \). Petersen \( (49) \) has shown in another vole species, *Microtus caniculus*, that neither neonatal castration nor treatment with flutamide or ATD feminizes the later sexual behavior of males and that females treated neonatally with TP still display normal feminine sexual behavior as adults. These unusual effects of perinatal hormones on sexual differentiation are not pervasive for every system throughout the entire genus *Microtus* because sexual behavior in female prairie and pine voles can, in some cases, be defeminized and masculinized by doses of TP \( (500–1000 \mu g) \) similar to those used in the present experiment \( (23, 24, 50) \). Although some components of sexual differentiation in prairie voles clearly differ from rats, voles are certainly not the only exceptions. Numerous other species, including domestic pigs, hamsters, ferrets, and some primates, respond to perinatal manipulations in gonadal hormones differently than laboratory rats \( (51) \), demonstrating that sexual differentiation occurs differently across species.

Considering that the TP treatment in females from experiment 4 extended from the first week after conception through the first week after birth, it is unlikely that our manipulations missed the critical period during which this system develops. Furthermore, our findings that neonatal castration demasculinized AVP expression in males and that neonatal estrogenic manipulations affected AVP in both sexes suggest that at least part of this critical period exists during early postnatal life. In rats, extrahypothalamic AVP mRNA expression is sexually dimorphic very early in development, beginning about 3 d after birth \( (52) \). It is not known when this system first becomes sexually dimorphic in prairie voles, but considering that prairie vole pups are precocial compared with rat pups and, at birth or within a few days after, have teeth and fur and are mobile \( (53–55) \), this system probably becomes sexually dimorphic early in development in voles, and it is likely that our TP treatments encompassed this period.

It might be suggested that prolonged treatment with exogenous androgens is somehow detrimental to this system, and we did find, in some cases, that a week-long treatment with 1000 μg TP was significantly or nonsignificantly feminizing in both sexes \( (\text{experiments 3, 4, and 7}) \). Gonadal hormones can down-regulate estradiol receptors in some areas of the developing and adult rat brain \( (56, 57) \), which could conceivably produce such a feminizing effect by not allowing sufficient estradiol receptor content to act upon. Nonetheless, repeated administration of T, DHT, lower doses of TP, or the single injection of 500 μg TP still did not masculinize the AVP system in females but was not feminizing.

In contrast to the lack of effects of exogenous TP, early postnatal manipulations of estrogenic activity with the es-
trogen receptor blocker TAM in males and the synthetic estrogen DES in females influenced the development of AVP expression. This indicates that, similar to rats, estrogen is a major masculinizing factor of this system in prairie voles. The variability in the effects of DES on AVP expression in the LS of females was unexpected. There is apparently an influence of the number of male siblings on this factor, and the simplest explanation is that females gestated in male-biased litters have greater in utero exposure to their brothers’ testicular hormones, which predisposes these females’ brains to be masculinized by neonatal exposure to estrogens. A similar litter sex ratio effect has been observed for the effects of neonatal DES on sexual differentiation of the preoptic area of rats (58). It may also be considered that differences in the effects of DES between females developing within male- or female-biased litters may not necessarily be due to prenatal factors but that postnatal interactions with a large number of brothers may influence females’ neural development, as well as their responses to gonadal hormones (59).

Although it has been suggested that the MA is one of the sources of the sexually dimorphic extrahypothalamic AVP pathway (7), we found no sex differences in AVP mRNA expression in rats. It is actually unclear whether this sex difference exists in rats (12, 60–62). The lack of a sex difference in the vole MA may indicate that the major contributor to sex differences in the terminal areas of the sexually dimorphic AVP projection (i.e., the LS and LHb) arises from the BST. It is also interesting to note that, although neonatal castration completely demasculinized AVP mRNA expression in the BST, it did not fully demasculinize AVP-ir in the terminal areas, indicating the lack of a one-to-one correspondence between AVP mRNA expression in the BST and AVP-ir in the LS and LHb.

At this point, we can only conjecture about what factors differ between rats and prairie voles that might prevent perinatal androgens from masculinizing the extrahypothalamic AVP system in the latter species. We believe that there are inherent species differences in the biology underlying sexual differentiation, although one may consider that differences in their laboratory environments may play a role. For example, many laboratory rats are fed only commercially available diets high in phytoestrogens, which can cross the placenta as well as later be incorporated in maternal milk (63). Early exposure to phytoestrogens modulates reproductive development and endocrine function in rodents (64, 65) and may (66) or may not (64, 67) influence sexual differentiation of their brain. Although some of the diet provided to our laboratory voles was a commercially available rabbit chow that may contain phytoestrogens, the majority of their diet was a mixture of hay, oats, corn, and sesame seeds that may be relatively low in phytoestrogens. Therefore, perinatal hormone manipulations may influence sexual differentiation in rats and voles differently because of inadvertent differences in endocrine factors present in the diets of their mothers. Nonetheless, biological differences between these species are likely important for differences in sexual differentiation. It may be possible that the differing sex chromosomes in male and female voles directly contribute to sex differences in this system (19). It could be that testicular hormones other than testosterone may be required to be present in order for exogenous androgens to masculinize the prairie vole brain. It could also be possible that T is not the primary hormone secreted by the neonatal prairie vole testes. This might indeed be the case, and preliminary data indicate that T is nondetectable in prairie voles of either sex when killed between 18–24 h after birth, whereas estradiol levels are higher in neonatal males than in females (Lonstein, J. S., and J. A. French, preliminary data). Under some conditions, the testes of some vole species can synthesize estradiol at relatively high levels, at least in adults (68). If this is found to be the case in neonates, an intriguing extension now being followed up would be that the perinatal prairie vole brain may not need to aromatize T into its estrogenic metabolites, and relatively little aromatase enzyme may exist in their developing brain.

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