

# 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  $\mu\text{g}$  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  $\mu\text{g}$ ), testosterone (100  $\mu\text{g}$ ), or dihydrotestosterone (200  $\mu\text{g}$ ) did not

masculinize AVP-ir in the female LS or LHb. In fact, 1000  $\mu\text{g}$  TP reduced it in some cases. However, 1000  $\mu\text{g}$  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)

EXTRAHYPOTHALAMIC EXPRESSION OF the neuropeptide arginine-vasopressin (AVP) or its nonmammalian analog arginine-vasotocin is sexually dimorphic in many vertebrate species, with males showing markedly greater AVP or arginine-vasotocin expression than females (1–6). In rats, this sexually dimorphic system originates in AVP-synthesizing cells of the bed nucleus of the stria terminalis (BST) and medial amygdala (MA) and terminates densely in the lateral septum (LS) and lateral habenula (LHb) (7, 8). Similar to almost all sex differences in the mammalian nervous system (9–11), differential exposure of developing males and females to gonadal hormones influences sex differences in extrahypothalamic AVP expression. For example, neonatal castration of male rats prevents the masculinization of AVP expression, whereas treating neonatal females with testosterone propionate (TP) masculinizes it (12–14). TP apparently requires aromatization into estradiol for much of its

effect (14), although the reduced androgen dihydrotestosterone (DHT) can also partly masculinize this system in rats if given in high doses (14).

As with many characteristics of the rodent brain, the influence of perinatal gonadal hormones in the generation of sex differences in extrahypothalamic AVP expression has been best studied in the laboratory rat. This is true even though sex differences in extrahypothalamic AVP expression exist in other rodent species (1, 15–19). It is virtually unknown in any other species whether sexual differentiation of this system relies on perinatal gonadal hormones in a manner similar to that in rats. It is quite possible that it does not because the mechanisms controlling sexual differentiation of other processes, including reproductive behaviors, can differ between species (20).

The prairie vole (*Microtus ochrogaster*) is an arvicoline rodent that is unusual among mammals because it shows many indices of monogamy, including pair bonding with mates and biparental behavior (21). They are a unique model to study sexual differentiation of behavior because some studies demonstrate that exposure to gonadal hormones during development influences their later sexual and parental behaviors differently than it does in rats. Unlike rats (22), neonatal castration does not demasculinize sexual behavior in male prairie voles and, in some cases, neonatal TP does not masculinize it in females (23, 24). Furthermore, neonatal castration demasculinizes parental responsiveness in virgin

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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 × 28 × 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 reports (25, 26), surrogate dams readily accepted foster pups, and the fostered pups in all cases had very high survival rates. Also similar to these previous reports, survival of neonatally manipulated pups raised by their own dams was invariably 100% in all experiments. All pups in a given litter received the same treatment. Subjects were weaned at 21 d of age and housed with their littermates in mixed-sex groups. When subjects were 30 d old, they were rehoused 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 adult gonadectomy or at death and were consistent within each experiment. One and, in a few cases, two pups within a given litter were used as subjects. All procedures were performed in accord with the acceptable standards for use of animals in research by the National Institutes of Health, the University of Massachusetts, and Michigan State University.

### 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 of age, they were anesthetized with an ip injection of ketamine (62.5 mg/kg), xylazine (7.5 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-cm SILASTIC 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-cm 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).

### 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

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 dioxide and decapitated. Brains were rapidly removed from the skull, flash-frozen in cold 2-methyl butane, and stored at –70 C until sectioning.

### Immunocytochemistry

Brains were cut into 40- $\mu$ m 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'-diaminobenzidine tetrahydrochloride, 0.04% ammonium chloride, 0.0004% glucose oxidase, 0.75 mM nickel ammonium sulfate, and 0.3%  $\beta$ -D-glucose. Sections were mounted on microscope slides, dehydrated, and coverslipped.

### In situ hybridization

Brains were thawed to approximately –17 C, and the entire BST and MA were cut into 20- $\mu$ m sections on a cryostat. Sections were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at –80 C until further processing. The *in situ* hybridization was performed similarly to that described previously in the De Vries laboratory (14). Plasmids containing the cDNA AVP sequence (pGEM3-AVPexCc) were obtained from Dr. Tom Sherman (Georgetown University, Washington, D.C.), and the vector was cut using *Eco*RI and transcribed with SP6 RNA polymerase and UTP. The riboprobe contained 293 bases that complemented the rat AVP mRNA. The probe was labeled at the 3'-end with [<sup>35</sup>S]dATP (NEN Life Science Products, Products, Boston, MA) using terminal deoxynucleotidyl transferase (Life Technologies Inc., Rockville, MD). Every third section through the BST and MA was processed. Slides were air dried at room temperature, fixed in 4% paraformaldehyde in PBS for 30 min, rinsed in PBS, and incubated for 10 min in a solution of 1.5% triethanolamine hydrochloride-0.9% sodium chloride (SSC)-0.25% acetic anhydride (pH 8.0). Slides were then rinsed for 2 min in 0.03 M sodium citrate in 0.3 M SSC. Slides were incubated in 70, 80, 95, and 100% ethanol for 2 min each, incubated in chloroform for 5 min, incubated again in 100 and 95% ethanol for 5 min each, and air dried.

Each slide was placed horizontally after applying 100  $\mu$ l of the hybridization buffer (2,000,000 cpm/100  $\mu$ l) consisting of 50% formamide, 10% dextan sulfate, 1 M Denhart's solution, 2 $\times$  SSC, 0.5 mg/ml yeast tRNA, and 0.1% sodium pyrophosphate in diethylpyrocarbonate-treated water. Slides were coverslipped with Parafilm (purchased from Fisher Scientific) and placed in a humidified incubator set at 37 C for approximately 14 h. Posthybridization washes consisted of 1 $\times$  SSC (4  $\times$  10 min each), 2 $\times$  SSC in 50% formamide (2  $\times$  20 min each at 52 C), 2 $\times$  SSC (2  $\times$  5 min each), RNase buffer (10 min at 37 C), RNase buffer with 100  $\mu$ g/ml RNase A (30 min at 37 C), 2 $\times$  SSC (2  $\times$  10 min each), 2 $\times$  SSC in 50% formamide (2  $\times$  20 min at 52 C), 1 $\times$  SSC (2  $\times$  5 min each), and 70% ethanol (2  $\times$  5 min each); and slides were then air dried. Slides were hybridized and coated with emulsion (NTB3, Kodak) and developed and fixed 2 wk later. Sections were then lightly counterstained with methyl green, dried, and coverslipped with glass microscope slides.

### Analysis of AVP fibers and cells

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

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  $450 \times 350 \mu\text{m}$  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. Immunocytochemical 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 *post hoc* 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  $P \leq 0.05$ .

## 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,  $P \leq 0.01$ ) 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 inter-

mediate between the control males and females ( $F_{(2,27)} = 9.19$ ,  $P \leq 0.001$ ), with all groups significantly different from each other, ( $P \leq 0.05$ ; Figs. 1 and 2). A similar pattern of differences between groups was also found in the LHb ( $F_{(2,28)} = 7.71$ ,  $P \leq 0.003$ ; 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 ( $\alpha, \alpha$ -trifluoro-2-methyl-4-nitro-m-propionoluidide; Sigma, St. Louis, MO) dissolved in 100  $\mu\text{l}$  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  $\mu\text{l}$  sesame oil beginning 1 wk after pairing with a male. To generate control subjects, other pregnant females ( $n = 13$ ) received two injections of 100  $\mu\text{l}$  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 then 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-

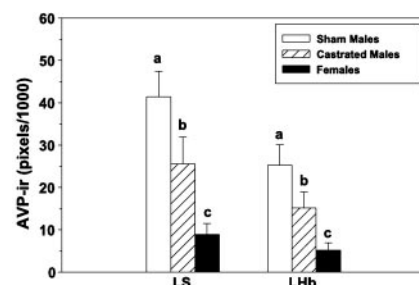
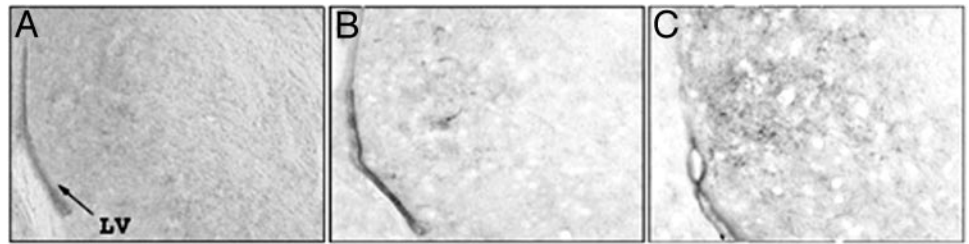


FIG. 1. AVP-ir (mean  $\pm$  SEM pixels covered by immunoreactive fibers) in the LS and LHb of female prairie voles and male prairie voles that received a neonatal sham surgery or neonatal castration. Different letters above bars indicate significant differences between groups within each brain region;  $P \leq 0.05$ .

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.



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 ( $F_{(2,32)} = 5.85$ ,  $P \leq 0.007$ ; 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 were castrated. 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.

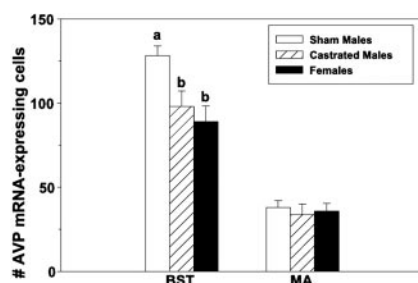


FIG. 3. Number (mean  $\pm$  SEM) of AVP mRNA-expressing cells in the BST and MA of female prairie voles and male prairie voles that received either a neonatal sham surgery or neonatal castration. Different letters above bars indicate significant difference between groups within each brain region;  $P \leq 0.05$ .

**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.

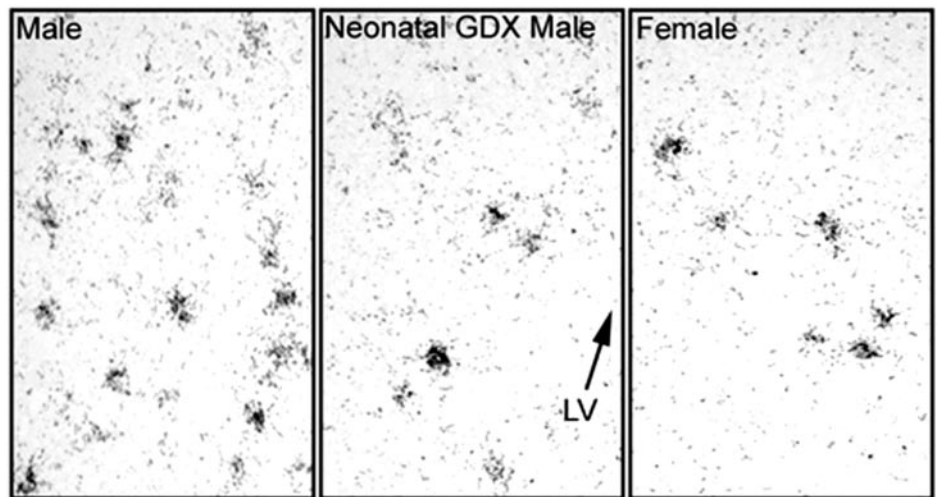


FIG. 4. Photomicrographs of AVP mRNA-expressing cells in the BST of a representative sham-operated control male, neonatally gonadectomized (GDX) male, and sham-operated control female. LV, Lateral ventricle.

#### 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  $\mu\text{g}$  TP diluted in 50  $\mu\text{l}$  sesame oil ( $n = 8$ ) or sesame oil ( $n = 8$ ). A group of males injected on postnatal d 3 with 50  $\mu\text{l}$  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  $\mu\text{g}$  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 ( $F_{(2,19)} = 32.83, P \leq 0.0001$ ) and LHb ( $F_{(2,19)} = 13.024, P \leq 0.0003$ ; 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 ( $t_{14} = 2.36, P \leq 0.04$ ) and LHb ( $t_{14} = 2.24, P \leq 0.05$ ).

#### 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  $\mu\text{g}$  TP

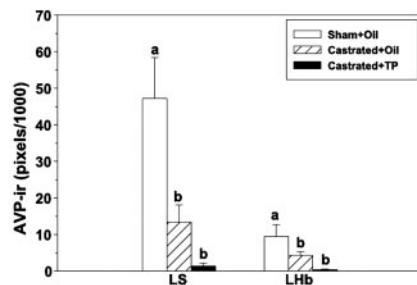


FIG. 5. AVP-ir (mean  $\pm$  SEM) in the LS and LHb of male prairie voles that received a sham surgery at birth and then treated with oil, males that were castrated at birth and then treated with oil, or males that were castrated at birth and received TP for 7 d. Different letters above bars indicate significant differences between groups with each brain region;  $P \leq 0.05$  ( $P \leq 0.053$  for LHb).

diluted in 50  $\mu\text{l}$  sesame oil ( $n = 9$ ) or sesame oil ( $n = 8$ ). A group of males injected on postnatal d 2, 5, 8, and 11 with 50  $\mu\text{l}$  sesame oil ( $n = 7$ ) was used as the positive control. Subjects were gonadectomized as adults, implanted with 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 ( $F_{(2,21)} = 9.31, P \leq 0.002$ ) and LHb ( $F_{(2,21)} = 6.54, P \leq 0.007$ ; Fig. 8).

#### Experiment 7: effects of daily neonatal administration of T, DHT, 75 $\mu\text{g}$ TP, or 1000 $\mu\text{g}$ TP on AVP-ir in females

**Methods.** Females were injected daily for the first week after birth with either 50  $\mu\text{l}$  sesame oil ( $n = 7$ ) or 100  $\mu\text{g}$  unconjugated T ( $n = 9$ ), 200  $\mu\text{g}$  DHT ( $n = 11$ ), 75  $\mu\text{g}$  TP ( $n = 10$ ), or 1000  $\mu\text{g}$  TP ( $n = 7$ ) diluted in 50  $\mu\text{l}$  sesame oil. A group of males injected the first week after birth with 50  $\mu\text{l}$  sesame oil ( $n = 10$ ) was used as the positive control. Subjects were gonadectomized as adults, implanted with a T-filled capsule, and killed 3 wk later. Brains were later processed for AVP immunocytochemistry.

**Results.** The LHb of one oil-treated female, one oil-treated male, and one DHT-treated female were either lost or dam-

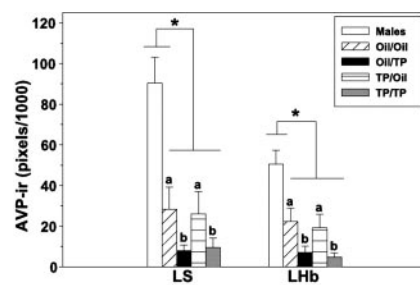


FIG. 6. AVP-ir (mean  $\pm$  SEM) in the LS and LHb of control male prairie voles and female prairie voles treated prenatally and/or postnatally with TP or oil. \*, Males significantly greater than all groups of females within each brain region,  $P \leq 0.05$ . Different letters above bars indicate significant differences between groups of females within each brain region;  $P \leq 0.05$ .

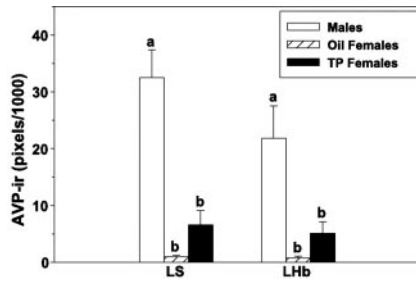


FIG. 7. AVP-ir (mean  $\pm$  SEM) in the LS and LHb of male and female prairie voles injected with 50  $\mu$ l sesame oil or females injected with 500  $\mu$ g TP once on postnatal d 3. Different letters above bars indicate significant differences between groups within each brain region;  $P \leq 0.05$ . Note that the differences between female groups are not statistically significant using a one-way ANOVA that includes males, but differences between female groups are significant when these two groups are compared with unpaired *t* tests.

aged and could not be analyzed. Administration of T, DHT, or either dose of TP for the first week of life had no effects on AVP-ir in the LS of females. All groups of females were statistically similar to each other and had significantly lower AVP-ir than control males, with the exception of T-treated females, which were not significantly different than any group ( $F_{(5,48)} = 2.51, P \leq 0.05$ ). These treatments also had no effect on AVP-ir in the LHb ( $F_{(5,45)} = 4.49, P \leq 0.003$ ; Fig. 9).

*Experiment 8: effects of neonatal tamoxifen (TAM) or diethylstilbestrol (DES) on AVP-ir*

**Methods.** Males were injected each day for the first week after birth with 50  $\mu$ l sesame oil ( $n = 11$ ) or 20  $\mu$ g of the estrogen receptor antagonist TAM dissolved in 50  $\mu$ l sesame oil ( $n = 9$ ). Females were injected each day for the first week after birth with either 50  $\mu$ l of sesame oil ( $n = 12$ ) or 1  $\mu$ g of the synthetic estrogen DES diluted in 50  $\mu$ l sesame oil ( $n = 11$ ). Subjects were gonadectomized during adulthood, implanted with T capsules, and killed 3 wk later. Brains were later processed for AVP immunocytochemistry.

**Results.** Males treated with TAM has significantly less AVP-ir in the LS than oil-treated males ( $t_{18} = 3.47, P \leq 0.003$ ), and TAM also tended to reduce AVP-ir in the LHb ( $t_{18} = 2.00, P = 0.061$ ; Fig. 10). Females treated neonatally with DES had significantly greater AVP-ir in the LS ( $U = 12.50, P \leq 0.001$ ), but DES did not significantly affect AVP-ir in the LHb ( $t_{21} = 0.50, P \geq 0.6$ ; Fig. 10). In addition, there was a significant

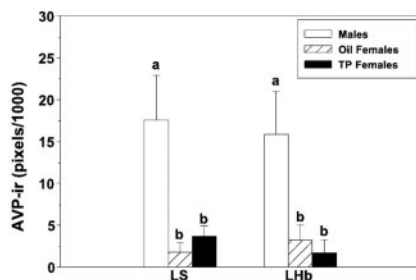


FIG. 8. AVP-ir (mean  $\pm$  SEM) in the LS and LHb of male and female prairie voles injected with 50  $\mu$ l sesame oil or females injected with 500  $\mu$ g TP on postnatal d 2, 5, 8, and 11. Different letters above bars indicate significant differences between groups within each brain region;  $P \leq 0.05$ .

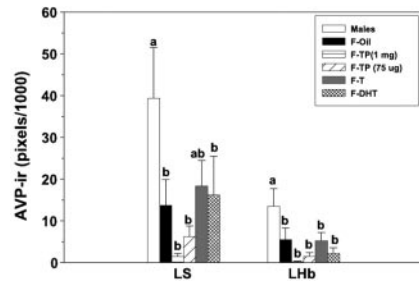


FIG. 9. AVP-ir (mean  $\pm$  SEM) in the LS and LHb of oil-treated control males, oil-treated control females, or females treated with TP (75 or 1000  $\mu$ g), T (100  $\mu$ g), or DHT (200  $\mu$ g) daily during the first week of life. Different letters above bars indicate significant differences between groups within each brain region;  $P \leq 0.05$ . F, Female.

polynomial regression between the levels of AVP-ir in the LS of DES-treated females and the sex ratio of their litter at birth ( $F_{(2,10)} = 4.98, P \leq 0.04$ ), 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  $P \leq 0.01$ ; data not shown). Treatment with TAM had no effect on male anogenital distance ( $t_{18} = 0.19, P \geq 0.8$ ). Adult body weight of males was not affected by any perinatal treatment in any experiment (all  $P \geq 0.1$ ; 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 ( $F_{(2,19)} = 100.1, P \leq 0.0001$ ; data not shown). In experiment

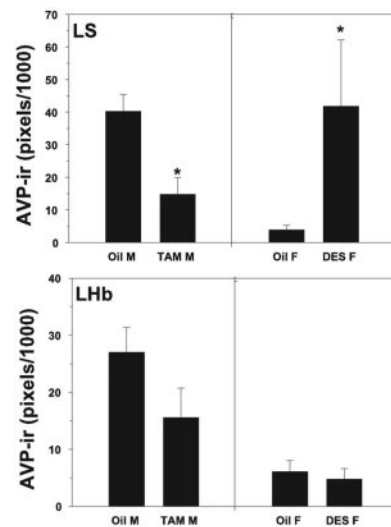


FIG. 10. AVP-ir (mean  $\pm$  SEM) in the LS (top) and LHb (bottom) of male prairie voles treated neonatally with oil or TAM (left) and of female prairie voles treated neonatally with oil or DES (right). \*, Significant differences between groups within sex within each brain region,  $P \leq 0.05$ .

5, injection of 500  $\mu\text{g}$  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  $\mu\text{g}$  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  $\mu\text{g}$  TP, 100  $\mu\text{g}$  T, and 200  $\mu\text{g}$  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 (all  $P \geq 0.5$ ; 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 (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 canicaudus*, 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  $\mu\text{g}$ ) 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  $\mu\text{g}$  TP was significantly or nonsignificantly feminizing in both sexes (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  $\mu\text{g}$  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 is 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 the MA. 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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### References

- Crenshaw BJ, De Vries GJ, Yahr P 1992 Vasopressin innervation of sexually dimorphic structures of the gerbil forebrain under various hormonal conditions. *J Comp Neurol* 322:589–598
- De Vries GJ, Buijs RM, Swaab, DF 1981 Ontogeny of the vasopressinergic neurons of the suprachiasmatic nucleus and their extrahypothalamic projections in the rat brain—presence of a sex difference in the lateral septum. *Brain Res* 218:67–78
- Boyd SK, Tyler CJ, De Vries GJ 1992 Sexual dimorphism in the vasotocin system of the bullfrog (*Rana catesbeiana*). *J Comp Neurol* 352:313–325
- Panzica GC, Castagana C, Viglietti-Panzica C, Russo C, Tlemcani O, Balthazart J 1998 Organizational effects of estrogens on brain vasotocin and sexual behavior in quail. *J Neurobiol* 37:684–699
- Foran CM, Bass AH 1999 Preoptic GnRH and AVP: axes for sexual plasticity in teleost fish. *Gen Comp Endocrinol* 116:141–152
- Moore RL, Richardson C, Lowry CA 2000 Sexual dimorphism in numbers of vasotocin-immunoreactive neurons in brain areas associated with reproductive behaviors in the roughskin newt. *Gen Comp Endocrinol* 117:281–298
- Miller M, De Vries GJ 1998 Anatomy and function of extrahypothalamic vasopressin systems in the brain. *Prog Brain Res* 119:3–20
- De Vries GJ 1990 Sex differences in neurotransmitter systems. *J Neuroendocrinol* 2:1–13
- Yahr P 1988 Sexual differentiation of behavior in the context of developmental psychobiology. In: Blass EM, ed. *Handbook of behavioral neurobiology*. Vol 9. New York: Plenum; 197–243
- Cooke BM, Hegstrom CD, Villeneuve LS, Breedlove MS 1998 Sexual differentiation of the vertebrate brain: principles and mechanisms. *Front Neuroendocrinol* 19:323–362
- Forger NG 2001 The development of sex differences in the nervous system. In: Blass EM, ed. *Handbook of behavioral neurobiology*. Vol 12. New York: Plenum; 153–208
- Wang Z, Bullock NA, De Vries GJ 1993 Sexual differentiation of vasopressin projection of the bed nucleus of the stria terminalis and medial amygdaloid nucleus in rats. *Endocrinology* 132:2299–2306
- Wang Z 1994 Testosterone effects on development of vasopressin messenger RNA expression in the bed nucleus of the stria terminalis and medial amygdaloid nucleus in male rats. *Brain Res Dev Brain Res* 79:147–150
- Han T, De Vries GJ 2003 Organizational effects of testosterone, estradiol, and dihydrotestosterone on vasopressin mRNA expression in the bed nucleus of the stria terminalis. *J Neurobiol* 54:502–510
- Buijs RM, Pevet P, Masson-Pevet M, Pool CW, De Vries GJ, Canguilhem B, Vivien-Roels B 1986 Seasonal variation in vasopressin innervation in the brain of the European hamster (*Cricetus cricetus*). *Brain Res* 371:193–196
- Hermes ML, Buijs RM, Masson-Pevet M, Pevet P 1990 Seasonal changes in vasopressin in the brain of the garden dormouse (*Eliomys quercinus L.*). *J Comp Neurol* 293:340–346

17. Bamshad M, Novak MA, De Vries GJ 1993 Sex and species differences in the vasopressin innervation of sexually naive and parental prairie voles, *Microtus ochrogaster* and meadow voles, *Microtus pennsylvanicus*. *J Neuroendocrinol* 5:247–255
18. Wang Z, Smith W, Major DE, De Vries GJ 1994 Sex and species differences in the effects of cohabitation on vasopressin messenger RNA expression in the bed nucleus of the stria terminalis in prairie voles (*Microtus ochrogaster*) and meadow voles (*Microtus pennsylvanicus*). *Brain Res* 650:212–218
19. De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP 2002 A model system for study of sex chromosome effects on sexual dimorphic neural and behavioral traits. *J Neurosci* 22:9005–9014
20. Baum MJ 1979 Differentiation of coital behavior in mammals: a comparative analysis. *Neurosci Biobehav Rev* 3:265–284
21. Carter CS, De Vries AC, Getz LL 1995 Physiological substrates of mammalian monogamy: the prairie vole model. *Neurosci Biobehav Rev* 19:303–314
22. Pfaff DW, Zigmond RE 1971 Neonatal androgen effects on sexual and nonsexual behavior of adult rats tested under various hormone regimes. *Neuroendocrinology* 7:129–145
23. Roberts RL, Zullo AS, Carter CS 1997 Sexual differentiation in prairie voles: the effects of corticosterone and testosterone. *Physiol Behav* 62:1379–1383
24. Smale L, Nelson RJ, Zucker I 1985 Neuroendocrine responsiveness to oestradiol and male urine in neonatally androgenized prairie voles (*Microtus ochrogaster*). *J Reprod Fertil* 74:491–496
25. Lonstein JS, De Vries GJ 2000 Influence of gonadal hormones in the development of parental behavior in adult virgin prairie voles (*Microtus ochrogaster*). *Behav Brain Res* 114:79–87
26. Lonstein JS, Rood BD, De Vries GJ 2002 Parental responsiveness is feminized after neonatal castration in virgin male prairie voles, but is not masculinized by perinatal testosterone in virgin females. *Horm Behav* 41:80–87
27. Wang Z, De Vries GJ 1993 Testosterone effects on paternal behavior and vasopressin immunoreactive projections in prairie voles (*Microtus ochrogaster*). *Brain Res* 631:156–160
28. Lonstein JS, De Vries GJ 2000 Sex differences in the parental behavior of rodents. *Neurosci Biobehav Rev* 24:669–686
29. Lonstein JS, De Vries GJ 1999 Sex differences in the parental behaviour of adult virgin prairie voles: independence from gonadal hormones and vasopressin. *J Neuroendocrinol* 11:441–449
30. Paxinos G, Watson 1998 *The rat brain in stereotaxic coordinates*. 4th ed. New York: Academic Press
31. Simerly RB 1990 Hormonal control of neuropeptide gene expression in sexually dimorphic olfactory pathways. *Trends Neurosci* 13:104–110
32. Breedlove SM, Cooke BM, Jordan CL 1999 The orthodox view of brain sexual differentiation. *Brain Behav Evol* 54:8–14
33. Cooke BM, Chowanadisai W, Breedlove SM 2000 Post-weaning social isolation of male rats reduces the volume of the medial amygdala and leads to deficits in adult sexual behavior. *Behav Brain Res* 117:107–113
34. Cooke BM, Tabibnia G, Breedlove SM 1999 A brain sexual dimorphism controlled by adult circulating androgens. *Proc Natl Acad Sci USA* 96:7538–7540
35. Becu-Villalobos D, Gonzalez Iglesias A, Diaz-Torga G, Hockl P, Libertun C 1997 Brain sexual differentiation and gonadotropins secretion in the rat. *Cell Mol Neurobiol* 17:699–715
36. Jacobson CD, Csernus VJ, Shryne JE, Gorski RA 1981 The influence of gonadectomy, androgen exposure, or a gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. *J Neurosci* 1:1142–1147
37. Del Abril A, Segovia S, Guillamon A 1987 The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth. *Brain Res* 429:295–300
38. Fitch RH, Cowell PE, Schrott LM, Denenberg VH 1991 Corpus callosum: demasculinization via perinatal anti-androgen. *Int J Dev Neurosci* 9:35–38
39. Nunez JL, Jurgens HA, Juraska JM 2000 Androgens reduce cell death in the developing rat visual cortex. *Dev Brain Res* 125:83–88
40. Vinader-Caerols C, Callado P, Segovia S, Guillamon A 2000 Estradiol masculinizes the posteromedial cortical nucleus of the amygdala in the rat. *Brain Res Bull* 53:269–273
41. Bakker J, Pool CW, Sonnemans M, Van Leeuwen FW, Slob AK 1997 Quantitative estimation of estrogen and androgen receptor-immunoreactive cells in the forebrain of neonatally estrogen-deprived male rats. *Neuroscience* 77:911–919
42. McAbee MD, DonCarlos LL 1999 Estrogen, but not androgens, regulates androgen receptor messenger ribonucleic acid expression in the developing male rat forebrain. *Endocrinology* 140:3674–3681
43. Quadros PS, Goldstein AY, De Vries GJ, Wagner CK 2002 Regulation of sex differences in progesterone receptor expression in the medial preoptic nucleus of postnatal rats. *J Neuroendocrinol* 14:761–767
44. Simerly RB, Swanson LW, Gorski RA 1985 Reversal of the sexually dimorphic distribution of serotonin-immunoreactive fibers in the medial preoptic nucleus by treatment with perinatal androgen. *Brain Res* 340:91–98
45. Simerly RB 1989 Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus. *Brain Res Mol Brain Res* 6:297–310
46. Siddiqui A, Shah BH 1997 Neonatal androgen manipulation differentially affects the development of monoamine systems in rat cerebral cortex, amygdala and hypothalamus. *Dev Brain Res* 98:247–252
47. Davis AM, Ward SC, Selmanoff M, Herbison AE, McCarthy MM 1999 Developmental sex differences in amino acid neurotransmitter levels in hypothalamic and limbic areas of rat brain. *Neuroscience* 90:1471–1482
48. De Vries GJ, Best W, Sluiter AA 1983 The influence of androgens on the development of a sex difference in the vasopressinergic innervation of the rat lateral septum. *Brain Res* 284:377–380
49. Petersen SL 1986 Perinatal androgen manipulations do not affect feminine behavioral potentials in voles. *Physiol Behav* 36:527–531
50. Wekesa KS, Vandenberg JG 1996 Androgen exposure and reproductive behavior in an induced ovulator, the pine vole (*Microtus pinetorum*). *Horm Behav* 30:416–423
51. Wallin K, Baum MJ 2002 Masculinization and defeminization in altricial and precocial mammals: comparative aspects of steroid hormone action. In: Pfaff DW, Arnold AP, Etgen AM, Fahrback SE, Rubin RT, eds. *Hormones, brain, and behavior*. Vol 4. New York: Academic Press; 385–424
52. Szot P, Dorsa DM 1993 Differential timing and sexual dimorphism in the expression of vasopressin gene in the developing rat brain. *Dev Brain Res* 73:177–183
53. Krukenberg SM, Gier HT, Dennis SM 1973 Postnatal development of the prairie vole, *Microtus ochrogaster*. *Lab Anim Sci* 23:53–55
54. Salo AL, Shapiro LE, Dewsbury DA 1994 Comparisons of nipple attachment and incisor growth among four species of voles (*Microtus*). *Dev Psychobiol* 27:317–330
55. Wang Z, Novak MA 1994 Alloprenatal care and the influence of father presence on juvenile prairie voles, *Microtus ochrogaster*. *Anim Behav* 47:281–288
56. DonCarlos LL, McAbee M, Ramer-Quinn DS, Stancik DM 1995 Estrogen receptor mRNA levels in the preoptic area of neonatal rats are responsive to hormone manipulation. *Dev Brain Res* 84:253–260
57. Lisciotto CA, Morrell JI 1993 Circulating gonadal steroid hormones regulate estrogen receptor mRNA in the male rat forebrain. *Brain Res Mol Brain Res* 20:79–90
58. Faber KA, Ayyash L, Dixon S, Hughes CL 1993 Effect of neonatal diethylstilbestrol exposure on volume of the sexually dimorphic nucleus of the preoptic area and pituitary responsiveness to gonadotropin-releasing hormone in female rats of known anogenital distance at birth. *Biol Reprod* 48:947–951
59. Morgan LR, Hite RF, Cushing BS 1997 Exposure to male siblings facilitates the response to estradiol in sexually naive female prairie voles. *Physiol Behav* 61:955–956
60. Wang Z, De Vries GJ 1995 Androgen and estrogen effects on vasopressin messenger RNA expression in the medial amygdaloid nucleus in male and female rats. *J Neuroendocrinology* 7:827–831
61. Al-Shamma HA, De Vries GJ 1996 Neurogenesis of the sexually dimorphic vasopressin cells of the bed nucleus of the stria terminalis and amygdala of rats. *J Neurobiol* 29:91–98
62. De Vries GJ, Al-Shamma HA 1990 Sex differences in hormonal responses of vasopressin pathways in the rat brain. *J Neurobiol* 21:686–693
63. Brown NM, Setchell KD 2001 Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. *Lab Invest* 81:735–747
64. Masutomi N, Shibusaki M, Takagi H, Uneyama C, Takahashi N, Hirose M 2003 Impact of dietary exposure to methoxychlor, genistein, or diisobutyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. *Toxicology* 192:149–170
65. Thigpen JE, Haseman JK, Saunders HE, Setchell KD, Grant MG, Forsythe DB 2003 Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. *Comp Med* 53:607–615
66. Faber KA, Hughes CL 1993 Dose-response characteristics of neonatal exposure to genistein on pituitary responsiveness to gonadotropin releasing hormone and volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in postpubertal castrated female rats. *Reprod Toxicol* 7:35–39
67. Weber KS, Setchell KD, Lephart ED 2001 Maternal and perinatal brain aromatase: effects of dietary soy phytoestrogens. *Brain Res Dev Brain Res* 126:217–221
68. Bilinska B, Schmalz-Fraczek B, Kotula M, Carreau S 2001 Photoperiod-dependent capability of androgen aromatization and the role of estrogen in the bank vole testis visualized by means of immunohistochemistry. *Mol Cell Endocrinol* 178:189–198