

Organizational Effects of Testosterone, Estradiol, and Dihydrotestosterone on Vasopressin mRNA Expression in the Bed Nucleus of the Stria Terminalis

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ABSTRACT: In adulthood, male rats express higher levels of arginine vasopressin (AVP) mRNA in the bed nucleus of the stria terminalis (BST) than do female rats. We tested whether this sex difference is primarily due to differences in neonatal levels of testosterone. Male and female rats were gonadectomized on the day of birth and treated with testosterone propionate (TP) or vehicle on postnatal days 1, 3, and 5 (P1, P3, and P5). Three months later, all rats were implanted with testosterone-filled capsules. Two weeks later, brains were processed for *in situ* hybridization to detect AVP mRNA. We found that neonatal TP treatment significantly increased the number of vasopressinergic cells in the BST over control injections. We then sought to determine the effects of testosterone metabolites, estradiol and dihydrotestosterone, given alone or in combination, on AVP expression in the BST. Rat pups were treated as described above, except that instead of testos-

terone, estradiol benzoate (EB), dihydrotestosterone propionate (DHTP), a combination of EB and DHTP (EB+DHTP), or vehicle was injected neonatally. Neonatal treatment with either EB or EB+DHTP increased the number of vasopressinergic cells in the BST over that of DHTP or oil treatment. However, treatment with DHTP also significantly increased the number of vasopressinergic cells over that of oil treatment. Hence, in addition to bolstering evidence that estradiol is the more potent metabolite of testosterone in causing sexual differentiation of the brain, these data provide the first example of a masculinizing effect of a nonaromatizable androgen on a sexually dimorphic neuropeptide system.

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INTRODUCTION

Since Phoenix and colleagues first demonstrated the masculinizing effect of prenatal testosterone (T) treatment on guinea pig sex behavior (Phoenix et al., 1959), the sex difference in T levels during develop-

ment has been recognized as a driving force of sexual differentiation of the mammalian central nervous system (Forger, 2001). In male rats, T secretion peaks from prenatal days 18–20 (Weisz and Ward, 1980) and again within hours after birth (Corbier et al., 1978), after which levels gradually diminish (Dohler and Wuttke, 1975). This early elevation in T levels coincides with a critical period within which T can permanently influence the development of behavior and certain neural structures (Cooke et al., 1998; Forger, 2001).

T is metabolized in the brain into estradiol (E), an estrogen, or into dihydrotestosterone (DHT), an androgen. Although nuclear receptors specific for estro-

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gen and androgen are present in the developing brain (Sheridan et al., 1974; DonCarlos and Handa, 1994; DonCarlos, 1996; McAbee and DonCarlos, 1998), E is the more potent metabolite in terms of masculinizing neural structure and behavior (Gorski, 1963; Kendrick and Drewett, 1980; Beyer, 1999; Hutchison, 2000). For example, perinatal treatment of rats with diethylstilbestrol, a synthetic estrogen, can masculinize the development of hypothalamic structures and reproductive behaviors (Dohler et al., 1984; Hines et al., 1987), whereas treatment of rats with DHT is by and large without effect on sexually dimorphic behaviors (van der Schoot, 1980). Masculinization of some sexually dimorphic features, such as the spinal nucleus of the bulbocavernosus, however, depends primarily on nonaromatizable androgen (Breedlove and Arnold, 1981; Nordeen et al., 1985). Perinatal DHT treatment also masculinizes aggressive and play behaviors in rats (Meaney and Stewart, 1981; Meaney et al., 1983), suggesting that androgenic effects on sexual differentiation of the central nervous system are not confined to the spinal cord.

Sexual differentiation of AVP expression in the bed nucleus of the stria terminalis (BST) is a process known to depend on T levels after birth. In adulthood male rats have twice as many vasopressinergic cells in the BST and have correspondingly denser projections to the lateral septum than do female rats (De Vries and Buijs, 1983; van Leeuwen et al., 1985; Miller et al., 1989). Castrating male rats on the day of birth reduces the number of vasopressinergic cells in the BST to levels typical of females, and testosterone propionate (TP) treatment on postnatal day 7 (P7) restores it to intact male levels (Wang et al., 1993). However, T treatment on P7 of females failed to increase AVP expression to male levels. It is not known whether earlier T treatment can increase the number of cells expressing AVP in females. Moreover, it is not known whether T's masculinizing effects in the BST depend on estrogenic or androgenic effects.

We examined the effects of neonatal T treatment on AVP expression in the BST in both males and females. We found that TP treatment in the first week did indeed increase the number of cells expressing AVP mRNA in both males and females over that resulting from oil treatment. We then compared the effects of estradiol benzoate (EB), dihydrotestosterone propionate (DHTP), a combination of EB and DHTP (EB+DHTP), and oil treatment on the number of cells expressing AVP mRNA in the BST. We found that neonatal treatment with either EB or EB+DHTP significantly increased the number of cells expressing AVP mRNA in the BST over that with DHTP and oil treatment. In addition, we found that neonatal treatment with DHTP significantly in-

creased the number of cells expressing AVP mRNA over that with oil treatment.

METHODS

Animals

Three-month-old Sprague-Dawley rats (Taconic Labs, Germantown, NY) were housed in a controlled environment with a 14:10 light/dark cycle. Food and water were available *ad libitum*. Male and female rats were paired in hanging cages. We checked for sperm plugs beneath the cages the morning following pairing. Females in cages under which sperm plugs were found were removed and housed in plastic tubs with wood chips and paper bedding. All procedures described here were done according to a protocol approved by the University of Massachusetts, Amherst IACUC, and they conformed to NIH guidelines.

On the day of birth, rat pups were subjected to sham surgery or gonadectomy. Pups were anaesthetized by placing them on ice. Castrations were performed by removing the testicles via ventral incisions at the level of the hipbones. Ovariectomies were performed by removing the ovaries via bilateral dorsal incisions just posterior to the kidneys. No cauterizations were necessary for either type of gonadectomy. Sham surgeries involved all of the procedures in gonadectomies, except for the actual removal of gonads. Incisions were closed with one or two sutures. Following surgeries, pups were warmed underneath a lamp until they became mobile, at which point they were returned to their mothers. Also following surgeries, litters were culled as necessary to a maximum of 10 pups.

Neonatal Hormone Treatments

Two studies were performed. The first compared the effects of T treatment to those of oil treatment in gonadectomized males and females. The second compared the effects of EB and DHTP, given alone or in combination, along with effects of oil treatment. Sham-operated rats were used as an internal control.

Sham-operated rats were treated with sesame oil (0.05 mL) on P1, P3, and P5. Gonadectomized rats were treated with sesame oil (0.05 mL), TP (500 μ g in 0.05 mL sesame oil; Sigma), EB (100 μ g in 0.05 mL sesame oil; Sigma), DHTP (2 mg in 0.05 mL sesame oil; Steraloids, Inc., Newport, RI), or a combination of EB and DHTP shown to have masculinizing effects on neural development (Goldstein and Sengelaub, 1990; 100 μ g and 2 mg, respectively, in 0.05 mL sesame oil). All injections were delivered subcutaneously on P1, P3, and P5. Litters were comprised of a combination of the treatment groups. Treatment groups were marked by specific toe or tail clips.

Adult Hormone Treatments

Three months after birth, T levels in all rats were matched. Rats were anaesthetized with methoxyflurane (Metofane;

Mallinckrodt) and subcutaneously implanted with a Silastic capsule (1.5 cm id, 2.4 cm od, length 2.5 cm) filled with T. These implants are sufficient for initiating and maintaining AVP mRNA expression in the BST of adult rats (De Vries et al., 1994). Sham subjects were also gonadectomized prior to capsule implantation.

Brain Collection and Processing

Two weeks following capsule implantation, rats were briefly asphyxiated with carbon dioxide and rapidly decapitated. Blood was collected and plasma was stored at -70°C for T radioimmunoassay. Brains were removed, quickly frozen in cold 2-methylbutane, and stored at -70°C until sectioning. Brains were cryosectioned and 20 μm thick coronal sections through the BST were collected. Every fourth section was collected on a Fisher Plus slide. A maximum of four sections were mounted per slide.

Probe Preparation

Plasmid containing the cDNA sequence of AVP exon C (pGEM3-AVPexCc) was obtained from Dr. Thomas Sherman of Georgetown University. The plasmid was cut using EcoRI, and 1 μg of DNA was transcribed with SP6 RNA polymerase in the presence of 500 μM each of GTP, ATP, and CTP, and 12 μM of UTP (6 μM of ^{33}P -UTP and 6 μM of cold UTP) to generate a 293 bp cRNA. Following transcription, DNA was digested with DNaseI (Boehringer Mannheim, Indianapolis, IN), whereupon it was purified with a phenol-chloroform extraction and two ethanol precipitations. The probe was stored in 0.1% SDS at -80°C until use.

In Situ Hybridization

Slides were processed separately. *In situ* hybridization was performed using the following incubations:

1. 4% formaldehyde in phosphate buffered saline (PBS) for 30 min
2. PBS twice for 2 min each
3. 0.25% acetic anhydride dissolved in 0.1 M triethanolamine hydrochloride and 0.9% sodium chloride (pH 8.0) for 10 min
4. Two times concentrated standard saline citrate (2X SSC; 1X SSC = 0.15 M NaCl and 0.015 M sodium citrate)
5. 30% ethanol for 2 min
6. 50% ethanol for 2 min
7. 70% ethanol for 2 min
8. 80% ethanol for 2 min
9. 95% ethanol for 2 min
10. 100% ethanol for 2 min
11. Chloroform for 5 min
12. 100% ethanol for 2 min
13. 95% ethanol for 2 min

The slides were then air dried.

Sections were covered with 100 μL of hybridization buffer containing 2×10^6 cpm of ^{33}P -UTP radiolabeled probe. The slides were coverslipped and placed in a humidified incubator at 52°C for 16–18 h. The hybridization buffer contained 50% formamide, 2X SSC, transfer RNA (250 $\mu\text{g}/\text{mL}$), 1% sodium pyrophosphate, 10% dextran sulfate, Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 200 mM dithiothreitol, and 2×10^6 counts per minute of probe per slide.

Following hybridization, coverslips were removed by soaking slides in 1X SSC. Slides were rinsed twice in 1X SSC and incubated in the following posthybridization solutions:

1. 1X SSC, four times for 15 min each
2. 2X SSC/50% formamide at 52°C , twice for 20 min each
3. 2X SSC at room temperature, twice for 10 min each
4. RNase buffer [0.5 M NaCl, 0.01 M Tris, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] at 37°C for 10 min
5. RNase buffer containing 100 $\mu\text{g}/\text{mL}$ RNase A (Sigma) at 37°C for 30 min
6. 2X SSC, twice for 10 min each
7. 2X SSC/50% formamide at 52°C , twice for 20 min each
8. 2X SSC, twice for 10 min each

Slides were dipped in distilled water, dehydrated through an ethanol series, and air dried. Slides were hybridized and coated with emulsion (NTB3, diluted 1 to 2 in double-distilled water; Kodak). Emulsion-coated slides were developed two weeks after processing. Sections were developed (Kodak Dektol), fixed (Kodak Fixer), and counter-stained with 0.5% methyl green.

Analysis

Sections matching levels -0.30 to -0.80 mm from bregma (Plates 19–23 in Swanson, 1992) were examined. Five successive sections were analyzed for each animal by a researcher blind to slide identities. The number and positions of AVP mRNA positive sites within the BST were observed with dark field microscopy and recorded within camera lucida drawings using a 4X objective. The BST was defined laterally by the internal capsule, medially by the stria medullaris and the fornix, ventrally by the myelinated fibers of the lateral preoptic area, and dorsally by the septohypothalamic nucleus [Fig. 1(A)].

Plasma levels of T at decapitation were determined using a ^{125}I T radioimmunoassay (ImmuChem™ Coated Tube Kit, ICN). Animals in which T levels were less than 1 ng/mL were not included in the *in situ* analysis. The remaining animals had T levels ranging between 1 and 5 ng/mL.

Statistical Analysis

The total number of AVP-positive cells across five sections in both left and right sides of the BSTs were analyzed. The

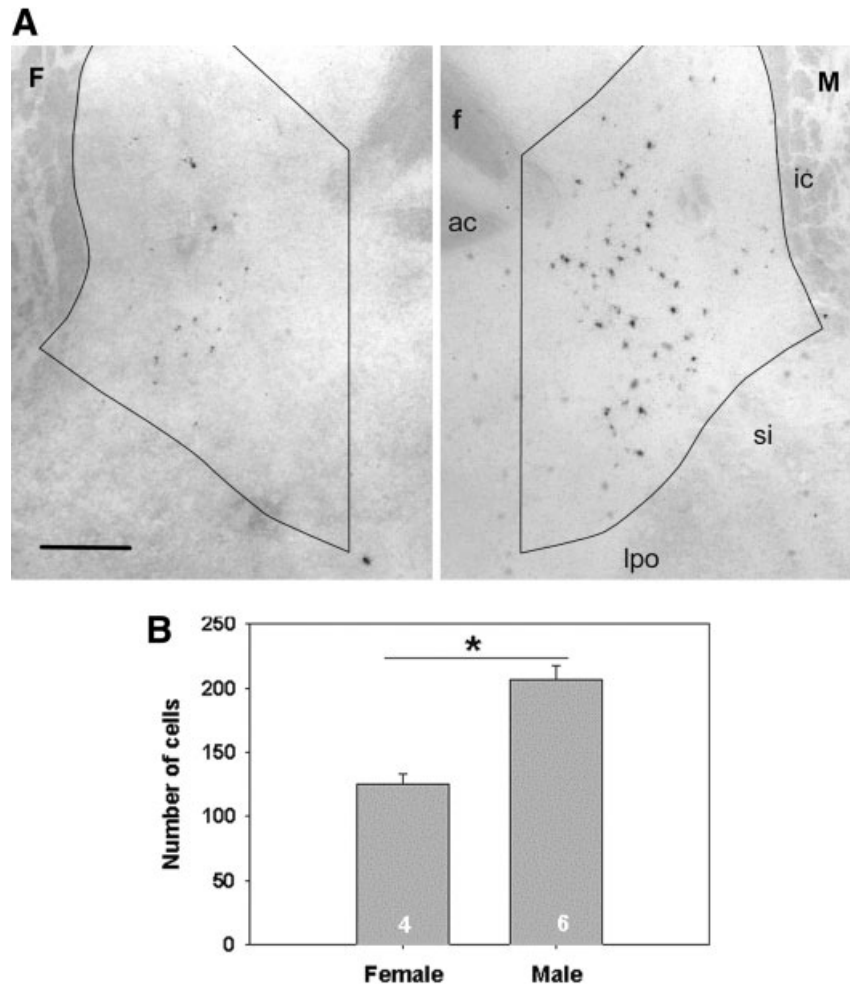


Figure 1 Rats that were sham gonadectomized neonatally exhibit sexually dimorphic AVP mRNA expression following implantation with testosterone-filled Silastic capsules in adulthood. (A) Photomicrograph of AVP-mRNA-positive cells in the BST of sham-operated control female (left) and male (right). Line delineates BST area analyzed. Scale = 500 μ m. ac, anterior commissure; ic, internal capsule; f, fornix; lpo, lateral preoptic area; si, substantia innominata. (B) The number of AVP-mRNA-positive cells in the BST of male and female rats is significantly different (Student's *t* test, $p < .0005$). Bars indicate means \pm S.E.M. Numbers within bars indicate number of animals in each group.

sex difference in sham-treated rats was evaluated using a Student's *t* test. The effects of TP and oil on the number of cells expressing AVP mRNA were analyzed with a two-way ANOVA with sex and TP treatment as variables. The effects of E and DHT were analyzed using three-way ANOVAs with sex, EB, and DHTP treatment as variables (male vs. female, EB or no EB treatment, DHTP or no DHTP treatment). Planned comparisons between groups were made with the Newman-Keuls posthoc test.

RESULTS

AVP mRNA Expression

The pattern of radiolabeled AVP mRNA in sham-operated males and females was consistent with that

reported previously (Miller et al., 1989). AVP mRNA expression was dense in the supraoptic nucleus and paraventricular nucleus (not shown). In the BST, AVP-mRNA-positive cells were dispersed throughout the structure [Fig. 1(A)]. There were more AVP-mRNA-expressing cells in the BST of males than of females (Student's *t* test; $p < .0005$) [Fig. 1(B)].

Neonatal TP Effects

Neonatal TP treatment significantly changed the number of AVP-mRNA-expressing cells in the BST of neonatally gonadectomized rats [ANOVA; $F(1, 32) = 16.57$; $p < 0.0001$]. There were no overall sex differences nor was there an interaction between sex

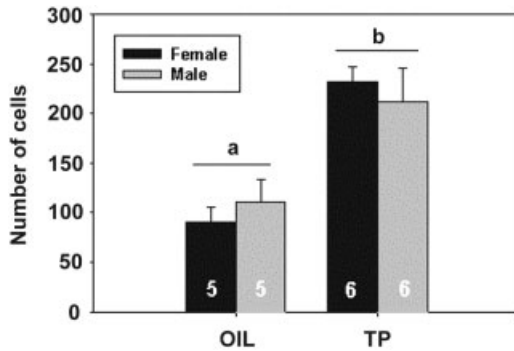


Figure 2 Number of AVP-mRNA-positive cells in the adult BST of male and female rats that were neonatally gonadectomized and treated with either oil or TP. Testosterone treatment significantly increased the number of cells expressing AVP mRNA over oil treatment (ANOVA, $p < .0001$). Different letters above bars designate significantly different groups (Newman-Keuls multiple-comparison posthoc test, $p < .05$).

and treatment. Posthoc tests revealed that TP treatment significantly increased the number of cells expressing AVP mRNA by roughly twofold compared to the number in oil-treated rats (Figs. 2 and 3).

Effects of Neonatal Estrogen and Androgen Treatment on AVP Expression

Neonatal EB as well as DHTP treatment significantly changed the number of cells expressing AVP mRNA in the BST [ANOVA; $F(1, 68) = 117.54$; $p < .000001$ and $F(1, 68) = 15.01$; $p < .0005$, respectively] (Fig. 4). Moreover, there was a significant interaction between EB and DHTP treatment [$F(1, 68) = 9.34$; $p < .005$]. There were no overall sex differences, nor were there any interactions between sex and treatment. Posthoc analysis demonstrated that EB as well as EB+DHTP treatment increased the number of cells expressing AVP mRNA by over twofold compared to oil treatment, while DHTP treatment increased the number of cells expressing AVP mRNA by about 40% over that of oil treatment.

DISCUSSION

Our results suggest that at birth male and female rats have the same potential to express AVP mRNA in the BST in adulthood. Neonatal TP treatment significantly increased AVP mRNA expression in the BST in neonatally gonadectomized males and females to a level comparable to that of sham-operated males, whereas oil treatment yielded levels comparable to those of sham-operated females. Our results suggest

further that T can influence the masculinization of AVP mRNA expression by estrogenic mechanisms because neonatal treatment with EB significantly increased AVP mRNA expression in the BST of gonadectomized males and females. However, T may masculinize AVP expression by androgenic mechanisms as well because DHTP treatment also significantly increased AVP expression. This is, therefore, the first demonstration of an organizational effect of a nonaromatizable androgen on a specific neurotransmitter system in the brain.

Our results do not allow us to conclude that the two sexes are equally responsive to gonadal steroid hormones as we used only one, relatively high, dose for each hormone. At birth, males have already experienced a pulse of T resulting in partially differentiated structures such as the spinal nucleus of the bulbocavernosus (Breedlove and Arnold, 1983; Nordeen et al., 1985). In addition, the prenatal surge in T may likewise bias developing neurons to express AVP in response to lower postnatal T levels than the ones we administered, such that given lower doses of hormones, an effect of sex may be observed.

The masculinizing effects of EB on AVP mRNA expression in the BST resembles E's effects on other sex differences in the brain. For example, neonatal EB treatment masculinizes cell number in regions such as the anteroventral periventricular nucleus and the central portion of the medial preoptic nucleus, and it increases the amount of galanin mRNA expressed per cell in gonadotropin releasing hormone neurons of the arcuate nucleus to levels found with TP treatment (Simerly, 1989; Merchenthaler et al., 1993; Arai et al., 1994, 1996; Simerly et al., 1997). Thus, our results provide further support for the idea that T's aromatization to E in the brain is a primary process in masculinization of the central nervous system. Although EB treatment influenced AVP mRNA expression more than DHTP treatment did, and DHTP treatment did not increase the number of AVP-mRNA-expressing cells in EB-treated rats, we cannot conclude that E is a more potent metabolite than DHT is for differentiation of AVP mRNA expression. Because we used only one dose for each testosterone metabolite, we cannot exclude the possibility that a higher dosage of DHTP would have caused more masculinization of AVP mRNA expression. Likewise, we cannot exclude the possibility that lower dosages of EB would have allowed us to see a cumulative effect of both steroids on AVP mRNA expression.

The effects of neonatal DHTP treatment on the number of cells expressing AVP mRNA in the BST suggest a more prominent role of DHT in the differentiation of neural structure than what is often assumed. Our data suggest that androgenic stimulation

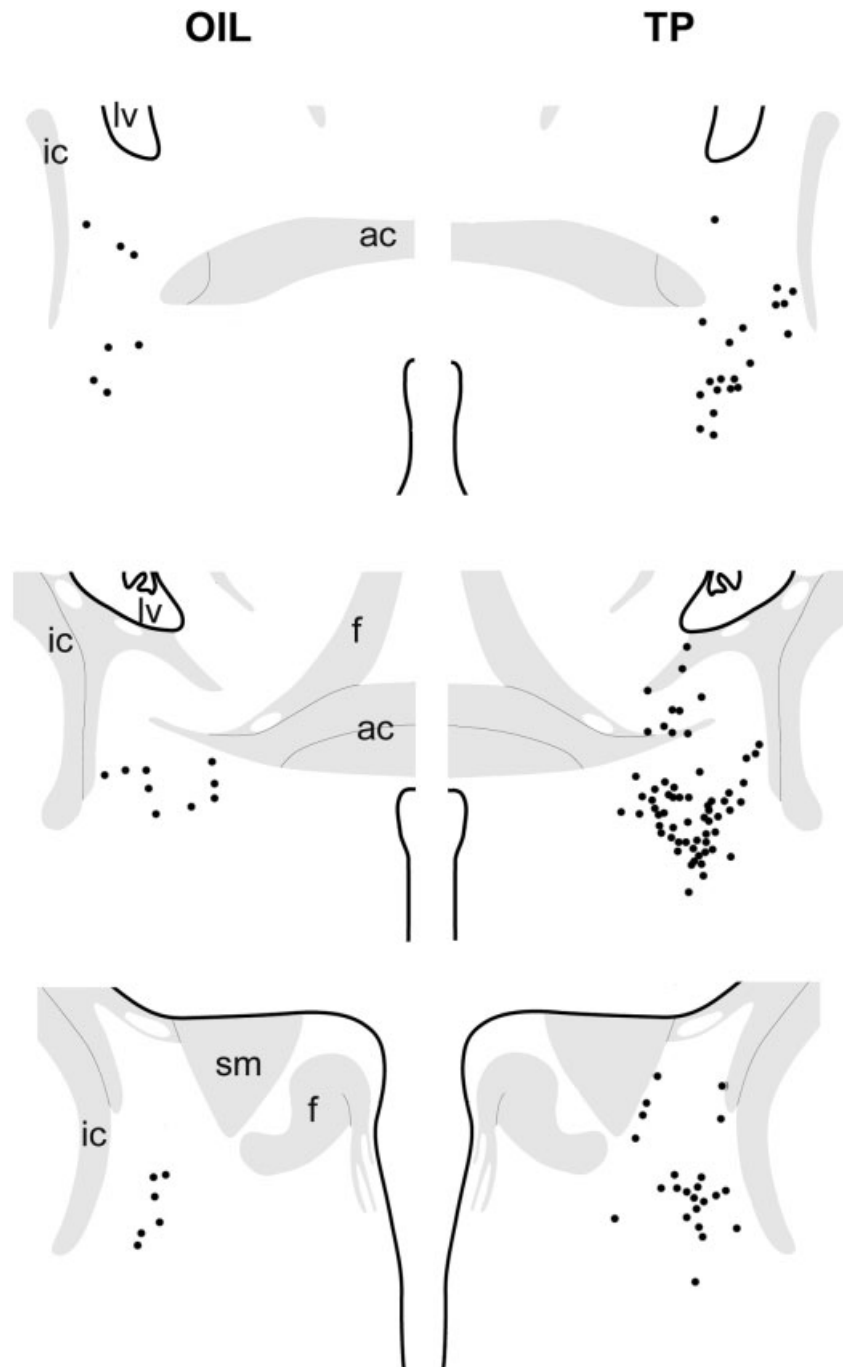


Figure 3 Schematic distribution of AVP-mRNA-positive cells in the BST in three representative coronal sections (Swanson, 1992; plates 19, 20, and 22). Relative distribution of cells within the BST was based on camera lucida drawings. Distribution of cells expressing AVP mRNA in oil-treated rats is on the left, and the distribution of cells expressing AVP mRNA in TP-treated rats is on the right side. ac, anterior commissure; f, fornix; ic, internal capsule; lv, lateral ventricle; sm, stria medullaris.

is at least sufficient to induce AVP expression in a subset of potentially vasopressinergic cells. In contrast to E's effect on sexual differentiation, few examples exist demonstrating an effect of DHT on sex-

ual differentiation. Some sexually dimorphic behaviors, such as play behavior, spatial memory, and escape behavior are influenced by neonatal exposure to androgen (Meaney and Stewart, 1981; Meaney,

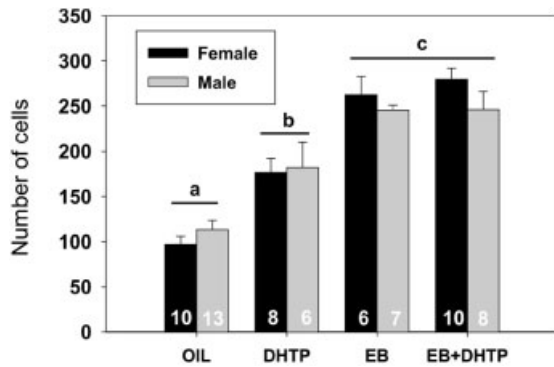


Figure 4 Neonatal treatment with EB, DHTP, or EB+DHTP increases the number of cells expressing AVP mRNA in the BST when compared with oil-treated controls. All subjects were gonadectomized neonatally. There was a significant effect of DHTP (ANOVA, $p < .0005$) and EB (ANOVA, $p < .000001$), as well as an interaction between the two (ANOVA, $p < .005$). Different letters above bars designate significantly different groups (Newman-Keuls multiple comparison posthoc test, $p < .05$). Bars indicate means \pm S.E.M. Numbers within bars indicate number of animals in each group.

1988; Isgor and Sengelaub, 1998). DHT promotes cell survival in the developing visual cortex (Nunez et al., 2000). In addition, neonatal DHTP can masculinize adult neuronal morphology in the CA3 region of the hippocampus (Isgor and Sengelaub, 1998). However, it is not known whether any of these sexually dimorphic systems play a role in the aforementioned androgen-sensitive behaviors.

The components for the direct action of estrogens and androgens on cellular differentiation in the BST are present neonatally. Aromatase, estrogen receptor alpha (ER- α), estrogen receptor beta (ER- β), and androgen receptor (AR) are expressed in the neonatal BST (Sheridan, 1984; Kuhnemann et al., 1994; Lauber and Lichtensteiger, 1994; Shinoda et al., 1994; DonCarlos et al., 1995; Yokosuka et al., 1997; Karolczak and Beyer, 1998; McAbee and DonCarlos, 1998, 1999; Shughrue et al., 1998). Whether ER- α , ER- β , or AR is expressed in neurons that will become vasopressinergic is not known.

Indirect effects of T and its metabolites may also contribute to the sexual differentiation of vasopressin expression in the BST. For example, the lateral septum, a major projection site for vasopressinergic neurons of the BST (De Vries and Buijs, 1983), contains a high density of ARs during development. Androgen may in fact alter the development of innervating BST neurons through target-dependent mechanisms as has been suggested for the projections from the BST to the anteroventral periventricular nucleus (Ibanez et al., 2001). Alternatively, neonatal hormone manipu-

lations may alter interactions among the developing neurons of the BST. E and T alter glia morphology in the hypothalamus (Mong and McCarthy, 1999), alter the activity of aromatase (Hutchison et al., 1991; Hutchison, 1997) and 5- α reductase in a region-specific manner (Melcangi et al., 1998), and influence GABAergic transmission in the developing brain (Perrot-Sinal et al., 2001), all of which may indirectly affect neurons in the same area, including cells that may potentially express AVP.

The masculinizing effects of TP and EB on AVP expression in females is consistent with the idea that gonadal steroid hormones induce AVP expression in a subset of galaninergic cells (Planas et al., 1995; Han and De Vries, 1999) rather than prevent vasopressinergic cells from undergoing programmed cell death. AVP expression in hormone-treated females was consistently indistinguishable from that in similarly treated males, which suggests that males and females have an equal number of potential vasopressinergic cells on the day of birth. For systems known to be strongly influenced by sex differences in programmed cell death such as the spinal nucleus of the bulbocavernosus (Breedlove and Arnold, 1983; Nordeen et al., 1985) and in the medial preoptic nucleus, irreversible sex differences in neuron number exist by the day of birth. (Dodson and Gorski, 1993; Arai et al., 1996; Davis et al., 1996; Jacobson et al., 1981). In these systems, complete masculinization of females requires pre- and postnatal hormone treatments.

Overall, our findings provide support for the idea that T influences the sexual differentiation of AVP mRNA by acting via androgenic and estrogenic mechanisms. Elucidation of the respective roles of E and DHT in sexual differentiation of AVP expression may aid in future studies addressing the role of this neuropeptide in sexually dimorphic behaviors, such as aggression (Compaan et al., 1993; Everts et al., 1997), play behavior (Meaney et al., 1981, 1983), and social recognition in rats (Dantzer et al., 1988; Bluthé et al., 1990, 1993).

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