0013-7227/93/1326-2299$03.00/0
Endocrinology
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Vol. 132, No. 6
Printed in U.S.A.

Sexual Differentiation of Vasopressin Projections of the Bed Nucleus of the Stria Terminals and Medial Amygdaloid Nucleus in Rats*

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ABSTRACT

The vasopressin-immunoreactive (AVP-ir) projections of the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA) are much denser in males than in females even if males and females are treated with similar amounts of testosterone. Previous studies have established that testosterone influences AVP-ir projections during development, but not whether these effects of testosterone were permanent. This study tested the effects of various hormonal manipulations during development on the ability of testosterone to influence the AVP immunostaining in cells of the BST and MA and of fibers in the lateral septum of adult rats.

In the first experiment, male rats that were castrated at 3 months of age (control males) had more AVP-ir cells in the BST and a higher density of AVP-ir fibers in the lateral septum than neonatally castrated male rats, whose cell numbers and fiber density did not differ from female rats that were ovariectomized neonatally or at 3 months of age (control females). This suggested that testicular secretions influence sexual differentiation of AVP-ir fiber pathways after birth. The second experiment showed that males castrated at the day of birth or at 1 week after birth had less AVP-ir cells in the BST and MA and a lower AVP-ir fiber density in the lateral septum than male rats castrated at the third week after birth or control males. This indicated that testicular secretions influenced the differentiation of AVP-ir pathways around postnatal day 7. This was further confirmed in the third experiment, in which testosterone propionate treatment at the seventh postnatal day significantly raised AVP-ir fiber density in the lateral septum of neonatally gonadectomized male and female rats and fully restored the number of AVP-ir cells in the BST of neonatally castrated males. Combined, these data suggest that testosterone levels around the seventh postnatal day determine the sexual differentiation of AVP-ir projections to the lateral septum. (*Endocrinology* **132:** 2299–2306, 1993)

IN rodents, the level of gonadal hormones determines the direction of sexual differentiation of centrally regulated functions and behaviors by influencing the development of specific neuronal systems in a restricted, critical period around birth (1, 2). The vasopressin-immunoreactive (AVP-ir) projections to brain areas such as the lateral septum and lateral habenular nucleus may be among the systems specifically targeted by gonadal hormones. These projections, which are about twice as dense in males as in females (3, 4), originate in the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA; 5, 6). Correspondingly, in males the BST contains about two to three times as many AVP-ir cells, or cells that can be labeled for AVP messenger RNA (mRNA), as it contains in females (4, 7, 8). As for the MA, no significant differences in AVP-ir cell number could be detected (4), whereas there are no reports addressing sex differences in the number of cells that could be labeled for AVP mRNA in this area.

An earlier study designed to test whether perinatal levels of gonadal hormones influence the differentiation of these AVP-ir projections indicated that gonadal hormones influence the staining of AVP-ir fibers in the lateral septum not only in the first but also in the third postnatal week (9). Since this was beyond the critical period of sexual differentiation, this suggested that the staining of AVP-ir pathways remains sensitive to gonadal hormones. Follow-up studies indeed showed that gonadectomy of adult animals eliminates AVP immunostaining of BST and MA cells and their projections and AVP mRNA labeling in BST cells (7, 10, 11–13). In retrospect, the earlier study did not answer the question as to whether perinatal levels of gonadal hormones influence the sexual differentiation of AVP-ir pathways, since its subjects were killed within 4 weeks after birth, which is considerably shorter than the time needed for the AVP immunoreactivity to completely disappear from the fibers after gonadectomy of adult animals—which takes more than 8 weeks (10). It was therefore impossible to distinguish between permanent and temporary effects of gonadal steroids.

Since males have more AVP-ir cells in the BST and a higher density of AVP-ir fibers in the projection areas of the BST and MA than females even after gonadectomy and subsequent treatment with testosterone in adulthood (4), the sex differences in these projections appear to depend on more than just the circulating levels of gonadal hormones. To reevaluate whether perinatal levels of gonadal hormones determine the sexual differentiation of AVP pathways, we compared the effects of a number of hormonal manipulations during development on the ability of testosterone to stimulate AVP staining in BST and MA cells and their projections in adulthood. First we studied the effects of neonatal castration to determine whether testicular secretions influence the sex-

Received November 24, 1992.
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* This work was supported by NIMH Grants 1RO1MH47538 (to G.J.D.).

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ual differentiation of AVP-ir pathways postnatally. Then we tested when during development castration can no longer affect differentiation of AVP-ir pathways. Finally we tested whether testosterone-propionate treatment during development can indeed masculinize the AVP-ir projections of the BST and MA of neonatally gonadectomized rats.

Materials and Methods

Exp 1

Male and female Long-Evans pups (Charles River Labs, Wilmington, MA) were gonadectomized (seven males and five females) or sham-operated (control animals; nine males and seven females) on the day of birth. The litters were culled until they contained eight pups, *i.e.* two pups of each of the four experimental groups. At 3 months of age, all control animals were gonadectomized, whereas the neonatally gonadectomized animals were sham-operated under chloropent anesthesia. At the same time, all subjects were implanted with Silastic capsules filled with testosterone (2.5 cm; 1.5 mm id, 2.4 mm od), which previously yielded plasma testosterone levels in the physiological range (10). Four weeks later, all animals were injected intracerebroventicularly with colchicine (80 μg/kg) under chloropent anesthesia. Two days later, they were deeply anesthetized with chloropent and perfused through the ascending aorta with 0.9% saline followed by 5% acrolein in 0.1 M phosphate buffer, pH 7.6. The brains were removed, and 50-μm transverse sections were cut with a vibratome. Free-floating sections were pretreated with 0.1% sodium borohydride for 15 min, rinsed 15 min three times in 0.05 M Tris-HCl, pH 7.6, containing 0.9% NaCl (Tris-NaCl), and then incubated with the following solutions: 1) Tris-NaCl with 0.3% Triton (Tris-Triton) and 20% goat serum, 15 min; 2) rabbit anti-AVP serum (ICN, Lisle IL) 1:8000 in Tris-Triton containing 2% goat serum (Tritrigo) for 1.5 h at 37 C; 3) Tritrigo, 15 min rinse three times at 37 C; 4) biotinilated goat-antirabbit in Tritrigo, 45 min at room temperature; 5) Tritrigo, 15 min rinse twice, followed by Tris-NaCl, one 15 min rinse; 6) ABC complex in Tris-NaCl, 45 min; 7) Tris-NaCl, 15 min rinse three times, and 8) 0.05% 3-3'-diamino-benzidine in Tris-NaCl with 0.0015% H_2O_2, 25 min. After three rinses in Tris-NaCl, sections were mounted on slides, air dried, and coverslipped. Specificity of the antiserum was tested by staining sections with anti-AVP serum that had been reacted with 50 μM AVP and by staining sections of the brains of homozygous Brattleboro rats, which produce an aberrant precursor of AVP. In both cases, no immunostaining was found in the areas studied.

AVP cells in the BST and MA were counted at the side of the colchicine injection in six consecutive sections that spanned the area with the highest number of AVP-ir cells (corresponding to plates 20–22 in the atlas of Paxinos and Watson (14) for the BST, and plates 28–30 for the MA). The density of AVP-ir fibers in the lateral septum was examined in the two consecutive sections that contained the highest fiber density (corresponding to plate 19 in the atlas of Paxinos and Watson; 14) by computerized gray-level thresholding using the IMAGE version 1.44 program developed by Dr. W. Rasbaud at NIH (15). The light intensity and camera setting were kept constant across the sections in order to standardize measurements. The density was expressed as the area covered by AVP-ir fibers in a 0.15 × 0.15-mm sampling area immediately bordering the lateral ventricle centered along the length of ventricular wall. Cell counts as well as analysis of fiber density were done in coded sections. The data were analyzed by two-way analysis of variance (ANOVA) with treatment and sex as between-subject variables. Significant interactions of treatment and sex were further evaluated using the Newman Keul's posthoc test.

Exp 2

Male Long-Evans rats were divided into four groups that were castrated at the day of birth (six animals), 1 week (eight animals), three weeks (eight animals), or 3 months (controls; five animals), respectively, under chloropent anesthesia. At the age of 3 months, all animals received testosterone implants. Four weeks later their brains were processed for AVP immunocytochemistry, after which the number of AVP-ir cells in the BST and MA were counted and the AVP-ir fiber density in the lateral septum was measured as described for Exp 1. The data were analyzed by one-way ANOVA, and significant treatment effects were further evaluated by the Newman Keul's posthoc test.

Exp 3

Male and female Long-Evans rat pups were gonadectomized (11 males and 10 females) or sham-operated (controls; 7 males and 6 females) at day of birth. On the seventh postnatal day, half of the neonatally gonadectomized animals (5 males and 5 females) were injected with testosterone propionate (Sigma, St. Louis, MO; 1 mg in 0.05 ml sesame oil per animal), whereas the remaining animals (6 males and 5 females) were injected with an equal volume of sesame oil. At the age of 3 months, all animals received testosterone implants. Four weeks later their brains were processed for AVP immunocytochemistry, after which the number of AVP-ir cells in the BST and MA were counted and the AVP-ir fiber density in the lateral septum was measured as described for Exp 1. The data were analyzed with two-way ANOVA with treatment and sex as the between-subject variables. Significant interactions of treatment and sex were further evaluated using the Newman Keul's posthoc test.

Results

Exp 1

Overall, animals that were gonadectomized neonatally had fewer AVP-ir cells in the BST ($F = 5.9$, df = 1,24, $P < 0.05$) and MA (F = 8.66, df = 1,24, $P < 0.001$) and a lower AVP-ir fiber density in the lateral septum ($F = 46.65$, df = 1,24, $P < 0.001$) than control animals. For the BST and lateral septum, however, these treatment effects differed by sex. Control males had about two times more AVP-ir cells in the BST ($F = 5.46$, df = 1,24, $P < 0.05$; Fig. 1) and about two to three times denser AVP-ir fiber plexus ($F = 12.94$, df = 1,24, $P < 0.001$; Fig. 2) than neonatally gonadectomized males or females of either hormonal condition, which did not differ from each other. No such interaction of sex and treatment was detected in the AVP-ir cells of the MA.

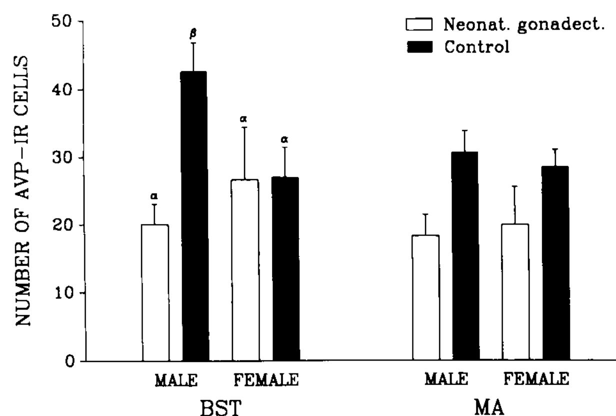

FIG. 1. Number of AVP-ir cells in the BST and MA. There was a significant interaction of treatment and sex for the number of AVP-ir cells in the BST (ANOVA, $P < 0.05$) but not in the MA. The symbols α and β show the results of the Newman Keul's posthoc test, which indicated that control males had more AVP-ir cells in the BST than all other groups. *Bars* indicate means ± SEM.

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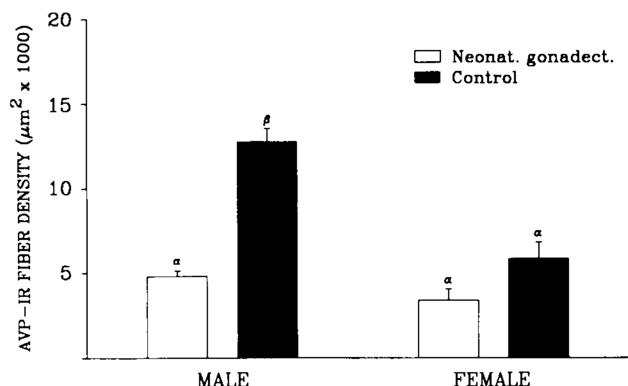

FIG. 2. AVP-ir fiber density in the lateral septum. There was a significant interaction of treatment and sex (ANOVA, $P < 0.001$). The symbols α and β show the results of the Newman Keul's posthoc test, which indicated that control males had a higher fiber density than all other groups. *Bars* indicate means ± SEM.

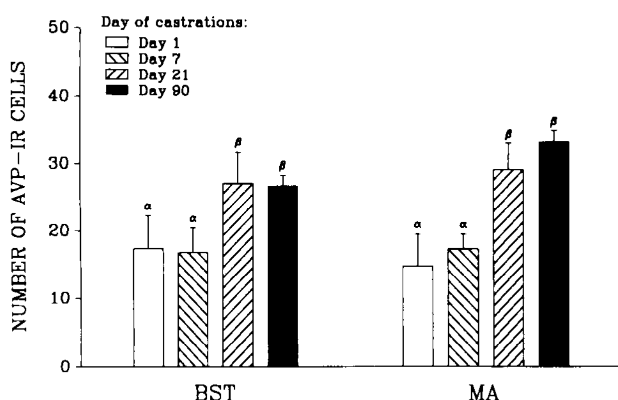

FIG. 3. Number of AVP-ir cells in the BST and MA. The time of castration significantly influenced the number of cells in both areas (ANOVA, $P < 0.05$ for BST and $P < 0.01$ for MA). The symbols α and β show the results of the Newman Keul's posthoc test, which indicated that control males and males castrated at the 21st postnatal day had more AVP-ir cells in the BST as well as the MA than males castrated earlier. *Bars* indicate means ± SEM.

Exp 2

Control males and males castrated at 3 weeks of age had higher numbers of AVP-ir cells in the BST and MA than males castrated at the day of birth or at postnatal day 7 (Figs. 3 and 4). When all four groups were analyzed separately, these differences were only significant in the MA ($F = 5.11$, df = 1,23, $P < 0.01$; Fig. 3). However, when the data of animals castrated at day 7 or earlier were combined and then compared with the combined data of animals castrated at 3 weeks or later, these differences were also significant in the BST ($F = 5.37$, df = 1,23, $P < 0.05$; Fig. 3).

Males castrated at postnatal day 7 had a lower AVP-ir fiber density in the lateral septum than males castrated at 3 weeks or control males but a higher fiber density than neonatally castrated males ($F = 27.0$; df = 1,23, $P < 0.001$; see Figs. 5 and 6). Males castrated at 3 weeks and control males did not differ from each other in fiber density.

Exp 3

Overall, hormonal treatment significantly altered the number of AVP-ir cells in the BST and MA ($F = 11.13$, df = 2,28, $P < 0.001$ for BST, and $F = 5.89$, df = 2,28, $P < 0.01$ for MA). The effects of hormonal treatment on the number of AVP-ir cells in the BST and MA, however, varied by sex. In the BST, the number of cells in neonatally castrated males was lower than the number in control males and neonatally castrated males injected with testosterone-proprionate on day 7, whereas in females no significant effects of the different hormonal treatments were detected ($F = 6.49$, df = 2,28, $P < 0.005$, Fig. 7). In the MA, the numbers of AVP-ir cells in both groups of neonatally castrated males was lower than in control males, whereas in females no significant effects of the different hormonal treatments were detected ($F = 4.32$; df = 2,28; $P < 0.05$, Fig. 8).

Overall, hormonal treatment significantly altered the density of AVP-ir fibers in the lateral septum ($F = 43.31$, df = 2,28, $P < 0.0001$). The effects of hormonal treatment on the density of AVP-ir fibers in the lateral septum also varied by sex. In males, the AVP-ir fiber density of neonatally gonadectomized animals that were injected with testosterone propionate on day 7 was significantly higher than in neonatally gonadectomized animals injected with vehicle but significantly lower than in control animals. In females, the AVP-ir fiber density of neonatally gonadectomized rats that were injected with testosterone propionate on day 7 was significantly higher than the fiber densities in neonatally gonadectomized and control females, which did not differ in fiber density from each other (F = 38.47, df = 2,28, $P < 0.001$, Fig. 9).

Discussion

This study indicates that the sexually dimorphic nature of the AVP-ir projections of the BST and MA depend on the levels of gonadal hormones during development. As reported previously (4), control males showed more AVP-ir cells in the BST and a higher density of AVP-ir fibers in the lateral septum than control females even though they received similar testosterone treatment. These differences were absent, however, in animals that were neonatally gonadectomized. In addition, since the AVP-ir fiber density in the lateral septum and the number of AVP-ir cells in the BST of neonatally gonadectomized animals equaled that of control females, testicular rather than ovarian secretions appear to be responsible for the sexual differentiation of the AVP-ir projections.

The effects of castration at various times after birth suggest that testicular secretions masculinize the AVP-ir projections of the BST and MA in the first weeks after birth. The number of AVP-ir cells in the BST and the AVP-ir fiber density in the lateral septum did not differ between animals castrated at 3 weeks after birth and control animals, whereas cell number and fiber density were lower in animals castrated earlier. Cell bodies and fibers, however, were differently affected by castration early in development. There were no clear differences in AVP-ir cell numbers in animals castrated

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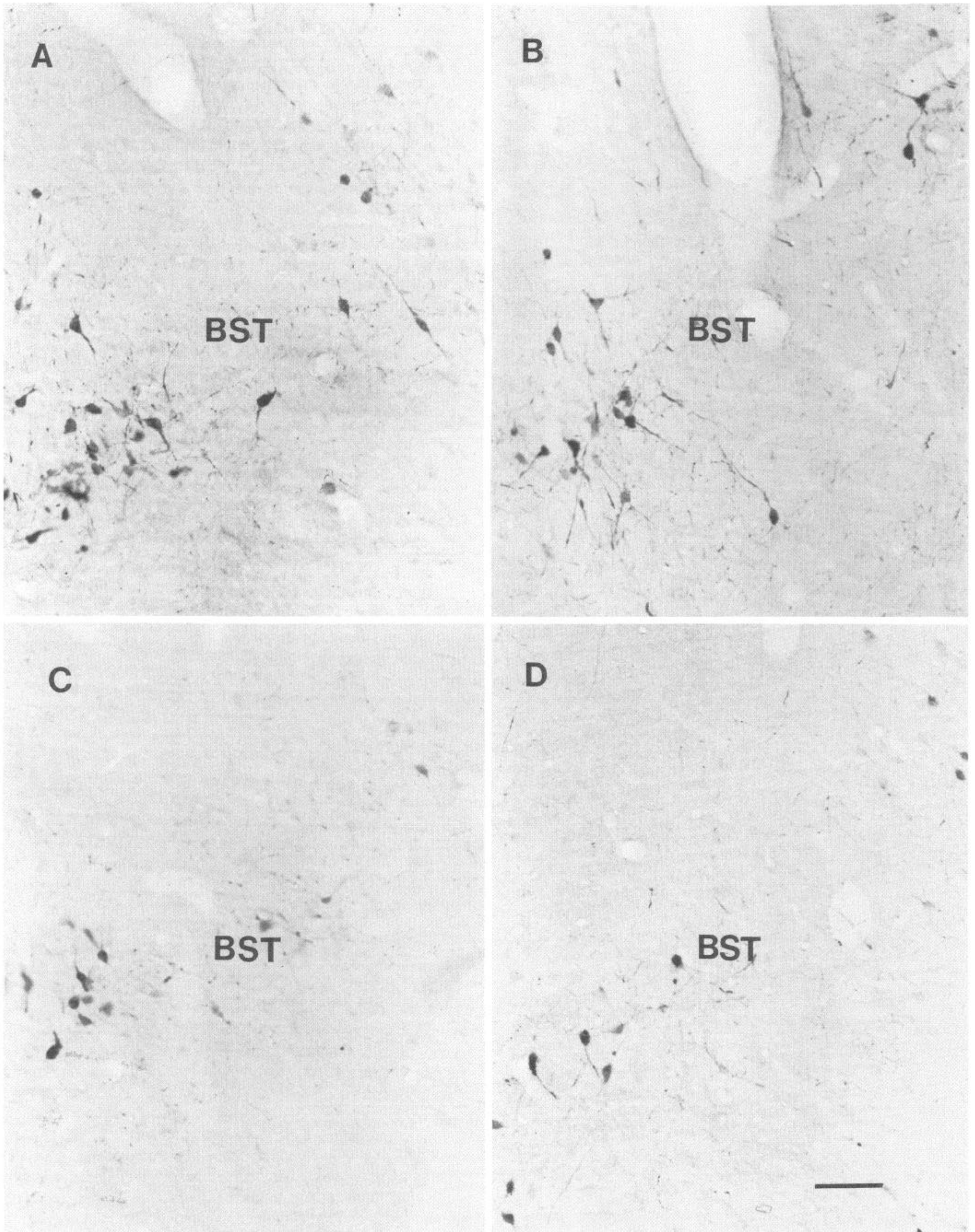

FIG. 4. Photomicrographs displaying AVP-ir cells in the BST of a control male (A), and males castrated at 3 weeks (B) and 1 (C) week after birth, or at birth (D). *Bar* = 50 μm.

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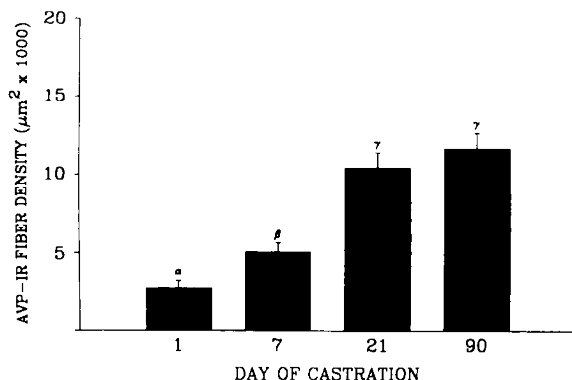

FIG. 5. AVP-ir fiber density in the lateral septum. The time of castration significantly influenced the fiber density (ANOVA, $P < 0.001$). The symbols α, β, and γ show the results of the Newman Keul's posthoc test, which indicated that control males and males castrated at the 21st postnatal day had a higher fiber density than males castrated at the 7th postnatal day, which, in turn, had a higher fiber density than males castrated at the 1st postnatal day. *Bars* indicate means ± SEM.

on postnatal days 1 and 7 in the BST and MA, which were comparable to those of control females. There were, however, differences in AVP-ir fiber density in the lateral septum, which was lower in animals castrated on postnatal day 1 than in animals castrated on postnatal day 7. These discrepancies suggest that testosterone acts on more than one cellular feature of AVP cells during differentiation.

The effects of testosterone propionate injections on the seventh postnatal day show the same discrepancy. These injections increase the number of AVP-ir cells in the BST to the level of control males, whereas they increased the AVP-ir fiber density in the lateral septum only to a level intermediate to that of control males and females. It may be that during development testosterone influences the number of cells that produce AVP independently from the level at which individual AVP-ir cells can produce AVP. Such a discrepancy in the developmental effects of testosterone on a sexually dimorphic system has also been found in the sexually dimorphic nucleus of the bulbocavernosus (16). Testosterone determines the number of motoneurons in this nucleus in the last week of pregnancy and the first week after birth, whereas it determines the size that the somata of these motoneurons will display in adulthood in the first 2 weeks after birth. As for the AVP-ir projections, testosterone may influence the density of the AVP-ir fibers in the first as well as the second and possibly the third week of life, whereas it determines the number of cells largely in the second and possibly the third week of life. An injection of testosterone propionate on the seventh postnatal day could in that case fully masculinize the AVP-ir cell number, but only partly the AVP-ir fiber density. Future studies in which testosterone is injected at different times after birth may reveal whether the differentiation of different sexually dimorphic features of the AVP-ir cells of the BST and MA have their own critical periods (*cf.* Ref. 16).

Interestingly, injections of testosterone propionate in females did increase the AVP-ir fiber density but not the number of AVP-ir cells in the BST of neonatally gonadectomized females. It may be that sex differences in prenatal gonadal hormone levels contribute differently to the developmental effects that testosterone will have postnatally on AVP-ir cell numbers and AVP-ir fibers. This possibility would also fit with the idea that more than one cellular feature of AVP cells underlie the differentiation of AVP cells.

There are indeed indications that more than one dimorphic cellular feature underlies the sex differences in the number of AVP-ir cells in the BST and the density of AVP-ir fibers in the lateral septum. When compared to female rats, male rats display more cells in the BST that can be labeled for AVP mRNA as well as a higher level of AVP mRNA labeling per cell (8). Such differences remain present even when males and females are gonadectomized and treated with similar levels of gonadal steroids (17). The difference in AVP mRNA labeling per cell may be the result of a lower responsiveness to androgens in females, since males and females treated with similar levels of estradiol hardly show a difference in AVP mRNA labeling per cell, whereas by adding dihydrotestosterone to the estradiol treatment one can increase the labeling per cell in males but not in females. Such differences may indeed be the result of sex differences in androgen receptor levels which have been reported for the BST and amygdala (18). Therefore, the sex difference in the number of AVP-ir cells may be caused by an absolute difference in the number of cells that can produce AVP, whereas the sex difference in the density of AVP-ir fibers in the lateral septum may be caused by a combination of sex differences in the number of cells that produce AVP and other factors, such as the aforementioned sex differences in the capacity of cells to synthesize AVP or sex differences in axonal branching as has been suggested for other sexually dimorphic systems (19).

The timing of the hormone effects allows for some inferences regarding cellular mechanisms underlying the sexual differentiation of AVP-ir cells. For example, since masculinization of these neurons appears to take place largely after birth, at which time practically all BST and MA neurons have been born in the areas that contain AVP-ir cells (20, 21), proliferation is an unlikely way for testosterone to influence the sexual differentiation of AVP cells. More likely, testosterone determines the number of AVP-ir cells by preventing naturally occurring cell death, as it appears to do in the differentiation of cells of the sexually dimorphic nucleus of the bulbocavernosus (22, 23). Alternatively, testosterone may influence a developmental decision as to whether BST and MA cells will produce AVP in adult animals. Interestingly, a recent report showed a surge of testosterone in males around postnatal day 7 that coincided with a surge in AVP mRNA labeling of BST cells in males but not in females, which showed neither a testosterone surge nor AVP mRNA labeling (24). Although these differences do not indicate by which cellular mechanism testosterone influences the differentiation of AVP cells, they suggests that around postnatal day 7 AVP cells in the BST are indeed stimulated in different ways in males and females.

In this and a previous study (4), no significant sex differences were found in the number of AVP-ir cells in the MA. Neither were there any effects of testosterone propionate

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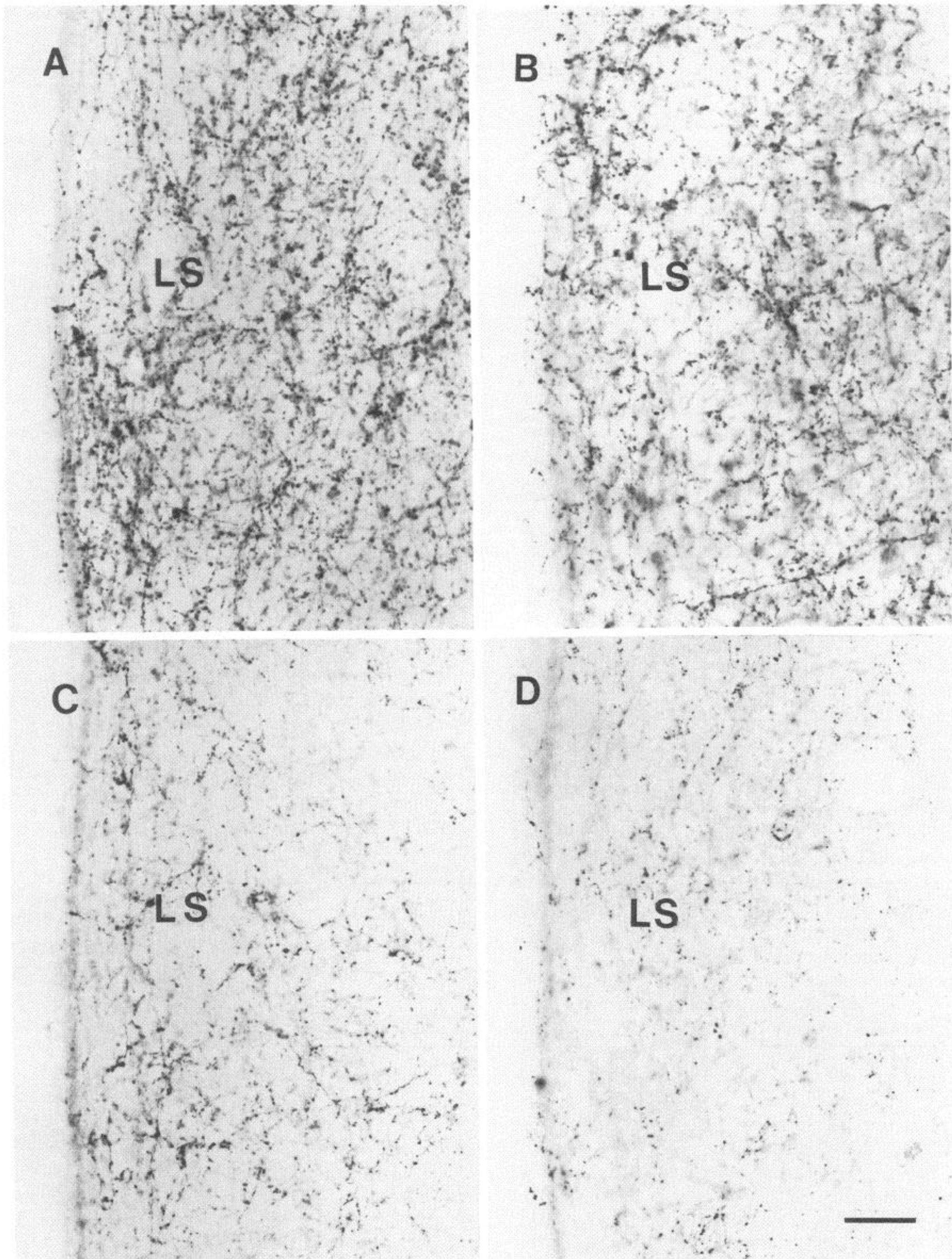

FIG. 6. Photomicrographs displaying AVP-ir fiber plexus in the lateral septum (LS) of a control male (A) and males castrated at 3 weeks (B) and 1 (C) week after birth, or at birth (D). *Bar* = 25 μm.

injections on the number of AVP-ir cells in the MA of neonatally castrated animals. However, males castrated neonatally and at postnatal day 7 had less AVP-ir cells in the MA than males castrated at 3 weeks after birth or control males, suggesting that the development of these cells is also influenced by gonadal steroids. It may be that sex differences and effects of neonatal manipulations in AVP-ir cells in the MA were less well detectable than in BST cells because the

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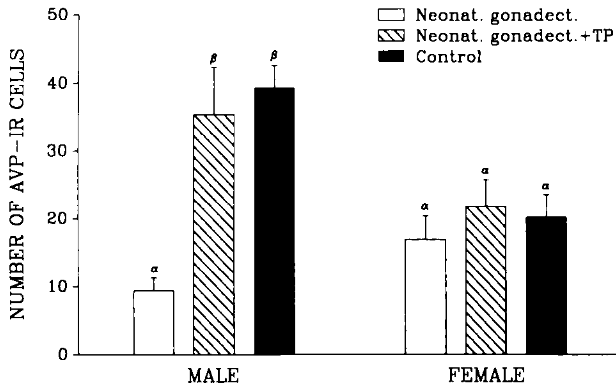

FIG. 7. Number of AVP-ir cells in the BST. There was a significant interaction of treatment and sex (ANOVA, $P < 0.005$). The symbols α and β show the results of the Newman Keul's posthoc test, which indicated that neonatally castrated males had lower numbers than neonatally castrated males injected with testosterone propionate (TP) or control males, which did not differ from each other, whereas no differences were found among the female groups. *Bars* indicate means ± SEM.

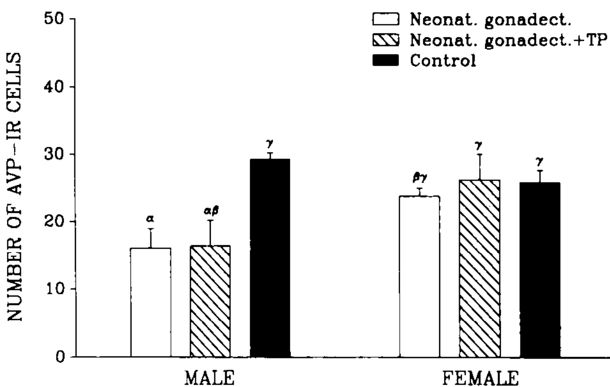

FIG. 8. Number of AVP-ir cells in the MA. There was a significant interaction of treatment and sex (ANOVA, $P < 0.05$). The symbols α, β, and γ show the results of the Newman Keul's posthoc test, which indicated that control males had higher numbers of AVP-ir cells than the other groups of males, whereas no differences were found among the female groups. *Bars* indicate means ± SEM.

variability in the number of AVP-ir cells was greater in the MA. This greater variability may be caused by a poorer accessibility of these cells to colchicine injected into the lateral ventricle. In the BST, which lies downstream of the site where colchicine was injected into the lateral ventricle, cell staining was typically enhanced at both sides of the brain, whereas in the MA cell staining was typically only enhanced at the side of the injection or not at all enhanced. Effects of neonatal manipulations on the number of cells in the MA might be better studied by labeling cells for AVP mRNA as was done to study sex differences in the BST of adult rats (8, 17).

The sex differences in AVP-ir projections of the BST and MA may contribute to the sex differences in functions such as sexual and aggressive behaviors and gonadotropin release, which are regulated by the BST and MA (25–28). In addition, such sex differences may contribute to the differential effects

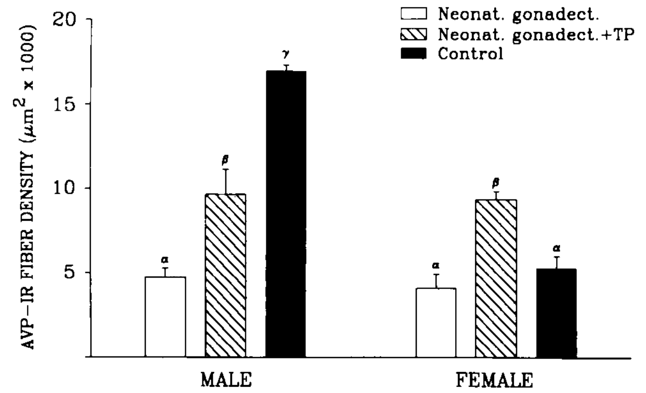

FIG. 9. AVP-ir fiber density in the lateral septum. There was a significant interaction of treatment and sex (ANOVA, $P < 0.001$). The symbols α, β, and γ show the results of the Newman Keul's posthoc test, which indicated that the AVP-ir fiber density was higher in males injected with testosterone propionate (TP) than in neonatally gonadectomized males but still lower than in control males, whereas the AVP-ir fiber density was higher in females injected with TP than in neonatally gonadectomized as well as control females. *Bars* indicate means ± SEM.

of AVP and AVP antagonists injections on functions such as social memory and sexual behavior (29–32). Given the clarity of the effects of hormonal manipulations during development upon the differentiation of AVP-ir projections of the BST and MA, this system appears to be well suited to study cellular events which may underlie sexual differentiation of behavior and other functions regulated by the brain.

Acknowledgments

The authors wish to thank Hussien Al-Shamma and Bradley Crenshaw for critically reading this manuscript.

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