Apoptosis during Sexual Differentiation of the Bed Nucleus of the Stria Terminalis in the Rat Brain

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ABSTRACT: **The bed nucleus of the stria terminalis (BST) in the rat forebrain differs between males and females. To test whether apoptosis may contribute to the development of sex differences in the BST, the incidence of apoptosis was determined in sham-treated males and sham-treated females sacrificed on postnatal days (PN) 2, 4, 6, 8, 10, and 12 (PN 1 being day of birth). More apoptotic nuclei were found in the principal nucleus of the BST (BSTpr) in females than in males, whereas the reverse was true for the lateral division of the BST (BSTl). Moreover, the volume of the BSTpr was larger in males than in females, whereas there was no sex difference in the volume of the BSTl. Our results also confirmed earlier reports indicating that the incidence of apoptosis in the central part of the medial preoptic nucleus (MPNc) is higher in females than in males. No sex difference in apoptosis was found in the ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN). The volume of the MPNc and VMH was larger in males than in females, whereas the PVN volume did not differ between males and females. To test whether sex differences in neonatal levels of gonadal steroids may cause sex differences in the incidence of apoptosis in the BSTpr, the incidence of apoptosis was compared between castrated males and females that were treated with testosterone propionate or vehicle on the day of birth. In the BSTpr of gonadal steroid-treated animals, the incidence of apoptosis was lower when compared to animals treated with vehicle, which was also true for the MPNc. These results are consistent with the hypothesis that gonadal steroids contribute to the sexually dimorphic differentiation of the BST by controlling the incidence of apoptosis.** © 2000 John Wiley & Sons, Inc. J Neurobiol 43: 234–243, 2000

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Several areas in the mammalian brain show robust sex differences in volume (Swaab and Hofman, 1995; Cooke et al., 1998). For example, in rats, the central part of medial preoptic nucleus (MPNc) is larger in males than in females (Gorski et al., 1978; Simerly et al., 1985), whereas the anteroventral periventricular nucleus (AVPv) is larger in females than in males (Bleier et al., 1982). Sexual differentiation of the MPNc and AVPv is regulated by perinatal levels of gonadal steroids (Jacobson et al., 1981a; Ito et al., 1986), which differ between males and females (Döhler and Wuttke, 1975; Weisz and Ward, 1980).

Gonadal steroids have been postulated to cause sex differences in volume by affecting developmental processes such as neurogenesis, neuromigration, apoptosis, or differentiation of cell phenotype (Arnold and Breedlove, 1985; Jacobson et al., 1985; Tobet et al., 1994). Of these, apoptosis and differentiation of

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cell phenotype appear to be major contributors to the development of sex differences found in the central nervous system. For example, the incidence of apoptosis in the rat spinal nucleus of the bulbucavernosus (SNB), MPNc, and bed nucleus of the accessory olfactory tract (BAOT) is higher in females than in males (Nordeen et al., 1985; Davis et al., 1996; Collado et al., 1998), whereas the reverse is true for the AVPv (Arai et al., 1994, 1996). Differentiation of cell phenotype appears to contribute to the sexually dimorphic development of, for example, the male nucleus of the preoptic area (MN-POA) of the ferret. The MN-POA is larger in males than in females (Tobet et al., 1986) mainly because male MN-POA cells are larger (Cherry et al., 1992).

Much is still unknown about the molecular and cellular mechanisms through which gonadal steroids induce sexual differentiation. Important obstacles for the identification of these mechanisms are the relatively small size of sexually dimorphic structures and the heterogeneity of the tissue in which they reside. An attractive area for such an analysis is the relatively large bed nucleus of the stria terminalis (BST), which contains a number of robust sex differences. For example, the principal nucleus of the BST (BSTpr) in rats is larger and has more cells in males than in females (Del Abril et al., 1987; Guillamon et al., 1988; Hines et al., 1992). Furthermore, the BST contains more immunoreactive cells for vasopressin, substance P, and cholecystokinin in males than in females (Van Leeuwen et al., 1985; Micevych et al., 1987; Malsbury and McKay, 1989; De Vries and Al-Shamma, 1990). The relatively large size of the BST was exploited by Hutton et al. (1998), who studied the development of connections between the BST, AVPv, and MPNc by implanting DiI crystals into the BST. This study showed that on postnatal day 11 males have dense projections from the BST to the AVPv and MPNc, whereas females have virtually none (Hutton et al., 1998).

In the present study, we examined the role of apoptosis during sexual differentiation of the BST. First, we tested whether the incidence of apoptosis in the BST differs between sham-treated males and sham-treated females. A similar analysis was performed in the sexually dimorphic MPNc, ventromedial nucleus of the hypothalamus (VMH) (Matsumoto and Arai, 1983), and in the non–sexually dimorphic paraventricular nucleus (PVN). Second, we tested whether sex differences in neonatal gonadal steroids can induce sex differences in the incidence of apoptosis in the BST and MPNc.

METHODS

Animals

Sprague Dawley rat pups were born of dams (Charles River Laboratories) mated in our own animal facilities. A 10 h light:14 h dark cycle was maintained throughout the study. Rat pups were sexed by examining the anogenital distance on postnatal day (PN) 1 (day of birth), and randomly placed in one of the following treatment groups: sham-treated, testosterone propionate–treated (TP), or vehicle-treated. On PN 1, male pups in the sham treatment group received a small skin incision located around the anogenital area, whereas male pups in the TP and vehicle treatment groups were gonadectomized. None of the female pups were gonadectomized. All males and females in the TP- and vehicle-treated groups were given a single subcutaneous injection of TP (1 mg/0.05 mL; Sigma Chemical Co, St. Louis, MO) or vehicle (sesame oil, 0.05 mL). All male and female pups were subjected to hypothermia anesthesia, marked by clipping of specific toes, and allowed to recover under a warm lamp before returning to the nest in litters of 8–12. To avoid litter effects, rat pups from the same litter were distributed across treatment groups. Pups were sacrificed on PN 2, 4, 6, 8, 10, or 12 ($n = 5$ per group; unless indicated differently in Results). In the first analysis, the incidence of apoptosis was compared between sham-treated male and sham-treated female rat pups. In the second analysis, the incidence of apoptosis was compared between TP- and vehicle-treated animals.

Brain Tissue Processing and Cresyl Violet Staining

Animals were anesthetized by means of hypothermia (PN 2–8) or chloral- hydrate/pentobarbital (PN 10–12) and decapitated. The brains were removed from the skull and immersion-fixed in 10% buffered formalin (Histoprep; Fischer Scientific, Pittsburgh, PA) for 3 days and stored in 0.05*M* Tris-buffered saline (TBS), pH 7.6, at 4°C. Brains were dehydrated in increasing grades of ethanol followed by toluene (Fischer Scientific) and embedded in paraffin (Fischer Scientific). Transverse serial sections (15 μm) were made with a rotary microtome and mounted on gelatine-coated glass slides. The sections were deparaffinized using HemoD (Fischer Scientific), rehydrated with decreasing grades of ethanol followed by Rho-purified H_2O, and stained with cresyl violet. After staining, sections were dehydrated and coverslipped using permount (Fischer Scientific).

Estimation of Total Volume

The boundaries of the BSTpr, lateral BST (BSTl), MPNc, VMH, and PVN were identified with the atlas of the developing rat brain by Alvarez-Bolado and Swanson (1996) using bright field microscopy (BH2 microscope; Olympus, Lake Success, NY). Digital images from every second sec-

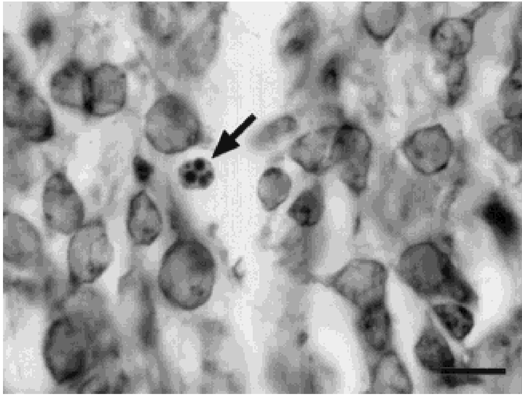

Figure 1 Representative photomicrograph of an apoptotic cell nucleus (arrow) in the rat forebrain. Scale bar, 10 μm.

tion through these structures were taken with a CCD72 camera (Dage; MTI, Michigan City, IN) attached to a Quick Capture frame grabber board (Data Translation, Marlboro, MA) in a Macintosh IIFx computer. The Scion IMAGE program v.1.57 developed by Dr. Rasband at the National Institutes of Health was used to measure the unilateral cross-sectional areas through the BSTpr, BSTl, VMH, and PVN. Bilateral cross-sectional measurements were made for the MPNc. The volume of each brain structure was calculated by multiplying the sum of the cross-sectional areas for each structure with 30 μm (i.e., the combined thickness of two sections) (Gundersen et al., 1988).

Estimation of Total Apoptosis

Apoptotic cell nuclei, identified by very intense staining, condensation, and often fragmentation of nuclear material, were counted in every second section at 400× magnification (Fig. 1). The total number of apoptotic cell nuclei in a brain area was estimated by multiplying the total number of apoptotic cell nuclei counted in all sections through this brain area by two (Königsmark and Murphy, 1970; Collado et al., 1998). The term "incidence of apoptosis" was used to indicate the total number of apoptotic cells per cubic micrometer. Because, this analysis assumes a uniform size of apoptotic profiles, a counting error between treatment groups could be induced, if the cross-sectional area of apoptotic profiles differed between the treatment groups. Therefore, the cross-sectional area (μm^2) of apoptotic profiles in the BST of males and females across treatment groups was measured on PN 6, on which a sex difference in the incidence of apoptosis was detected.

Statistical Analysis

The data of the first analysis were examined for significant differences in the incidence of apoptosis and volume of the BSTpr, BSTl, MPNc, VMH, and PVN during development with a two-way analysis of variance (ANOVA) with age and sex as between-subject variables. For the second analysis a three-way ANOVA was used with age, sex, and hormonal treatment as between-subject variables. A Student–Newman–Keuls test was used for *post hoc* analysis. Measurements of cross-sectional area of apoptotic profiles were tested for differences using a two-way ANOVA. Differences were considered significant if $p < .05$. All measurements were conducted by an observer who was blind to sex, treatment, and specific age of the subjects.

RESULTS

At all ages, we were able to recognize the different subdivisions of the BST as indicated in the atlas of Alvarez-Bolado and Swanson (1996). We could not analyze all sections of every area for each subject, because some sections were folded or torn. We have indicated below, in which cases less than five subjects per group were analyzed.

Principal Nucleus of the Bed Nucleus of the Stria Terminalis (BSTpr)

Four males were analyzed on PN 10 and three females on PN 12. Overall, females showed a higher incidence of apoptosis than males in the BSTpr [F (1, 45) = 19.18, $p < .0001$]. In addition, the incidence of apoptosis changed with age, peaking at PN 6 [F (5, 45) = 16.06, $p < .0001$), but these changes varied by sex [F (5, 45) = 7.43, $p < .0001$; Fig. 2(A)]. Post hoc analysis showed that on PN 6 female rat pups had a higher incidence of apoptosis than male rat pups ($p < .05$).

Overall, BSTpr volume was larger in males than in females [F (1, 45) = 17.78, $p < .0005$)]. In addition, BSTpr volume increased with age [F (5, 45) = 18.80, $p < .0001$], and this increase varied by sex [F (5, 45) = 4.28, $p < .005$; Fig. 2(B)]. Post hoc analysis revealed that the BSTpr volume was sexually dimorphic on PN 12 ($p < .05$).

Central Part of the MPN

Three males were analyzed on PN 2, four males on PN 6, four females on PN 4, and two females on PN 12. Overall, females showed a higher incidence of apoptosis than males in the central part of the MPN (MPNc) [F (1, 41) = 5.78, $p < .05$]. Although the incidence of apoptosis did not vary significantly by age, there was a significant interaction between age and sex [F (5, 41) = 4.49, $p < .005$; Fig. 2(C)]. Post hoc analysis showed that on PN 8, females had a significantly higher incidence of apoptosis ($p < .05$).

Overall, the MPNc was larger in males than in

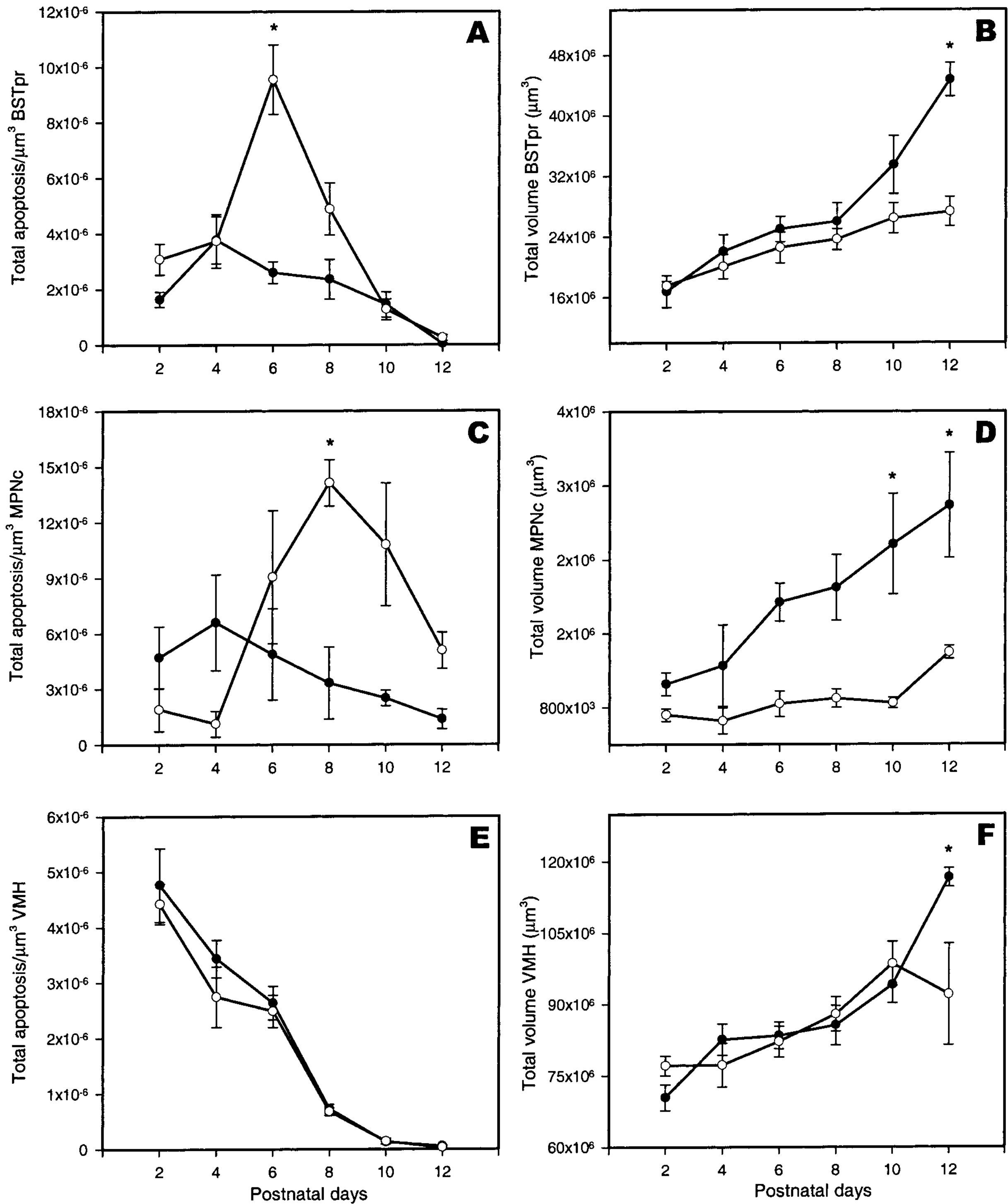

Figure 2 Graphs showing the incidence of apoptosis per total nucleus (A, C, E) and the volume of each nucleus (B, D, F) during postnatal development of the BSTpr (A, B), MPNc (C, D), and VMH (E, F). The incidence of apoptosis was higher in the BSTpr and MPNc of females (○) than males (●), whereas no sex difference in apoptosis was observed in the VMH. Bars, S.E.M. $*p < .05$.

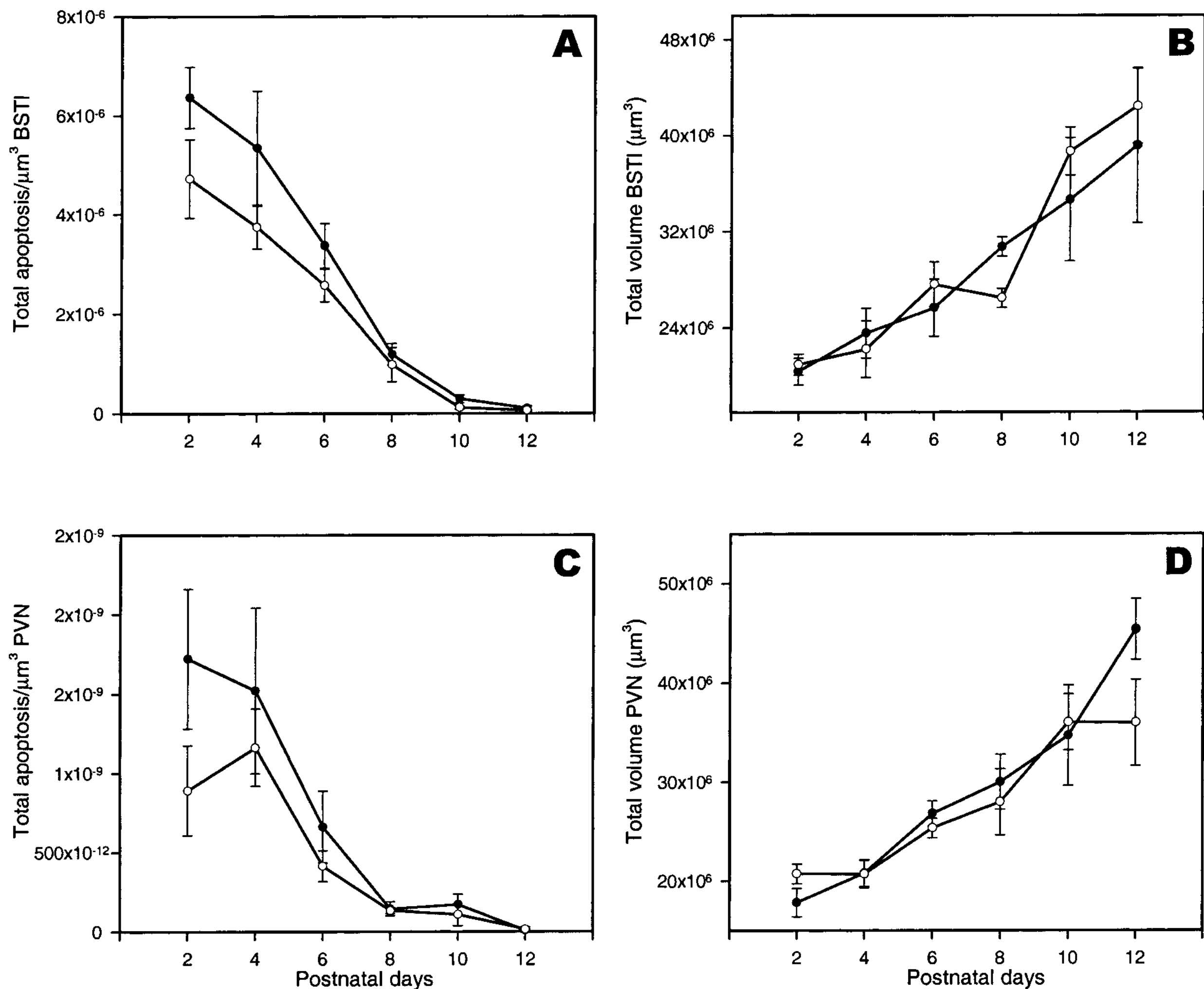

Figure 3 Graphs showing the incidence of apoptosis per total nucleus (A, C) and the volume of each nucleus (B, D) during postnatal development of the BSTl (A, B) and PVN (C, D). The incidence of apoptosis in the BSTl was higher in males (●) than in females (○). No sex difference in apoptosis was found in the PVN. In addition, there was no sex difference in the BSTl and PVN volume. Bars, S.E.M.

females [F (1, 41) = 29.23, $p < .0001$]. In addition, MPNc volume increased with age [F (5, 41) = 3.36, $p < .05$]. However, this increase did not vary by sex [Fig. 2(D)]. Post hoc analysis showed that on PN 10 and PN 12, males had a significantly larger MPNc than females ($p < .05$).

Lateral Division of the BST

Four males were analyzed on PN 2 and PN 10 and three females on PN 12. Overall males showed a higher incidence of apoptosis than females [F (1, 44) = 5.86, $p < .05$]. In addition, the incidence of apoptosis decreased with age [F (5, 44) = 36.74, $p < .0001$], but did not vary by sex [Fig. 3(A)].

Overall, the BSTl volume did not differ between males and females. However, BSTl volume increased with age [F (5, 44) = 13.37, $p < .0001$], but this increase did not vary by sex [Fig. 3(B)].

Ventromedial Hypothalamus

Four females were analyzed on PN 12. Overall, there was no sex difference in the number of apoptotic nuclei in the VMH. However, the incidence of apoptosis decreased with age [F (1, 47) = 66.9, $p < .0001$], but this decrease did not vary by sex [Fig. 2(E)].

Overall, the VMH volume did not differ between males and females, but it increased with age [F (5, 47)

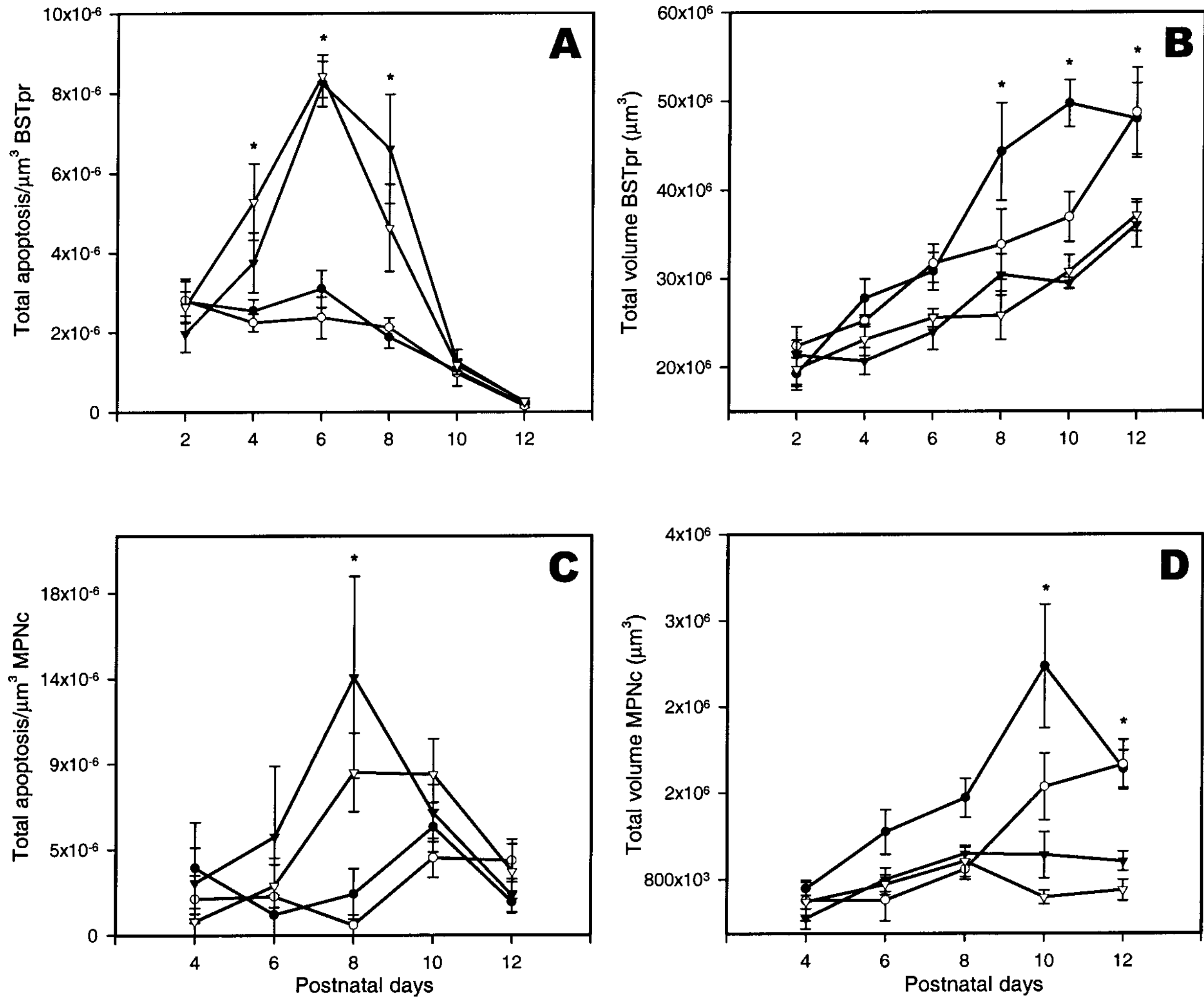

Figure 4 Graphs showing the incidence of apoptosis between postnatal days 2 and 12 in the BSTpr (A) and MPNc (C) and the volume of the BSTpr (B) and MPNc (D) in males (●, ▲) and females (○, △) treated with TP (circles) or vehicle (triangles). Note the suppressive effects of TP on the incidence of apoptosis in the BSTpr and MPNc and the increase in BSTpr and MPNc volume in animals treated with TP when compared to vehicle-treated animals. Bars, S.E.M. *$p < .05$ for treatment effect.

$= 14{,}1$, $p < .0001$], and this increase varied by sex [F (5, 47) = 3.2, $p < .009$; Fig. 2(F)]. The volume of the VMH increased between PN 10 to PN 12 in males, but not in females ($p < .05$).

Paraventricular Nucleus

Four females were analyzed on PN 12. Overall, there was no sex difference in the number of apoptotic nuclei in the PVN. However, the incidence of apoptosis decreased with age [F (5, 47) = 12.40, $p < .0001$], but this decrease did not vary by sex [Fig. 3(C)].

Overall, the PVN volume did not differ between males and females, but it increased with age [F (5, 47) = 18.81, $p < .0001$]. However, this increase did not vary by sex [Fig. 3(D)].

Gonadal Steroid Effects on Apoptosis: BSTpr

Overall, the incidence of apoptosis was significantly reduced in animals treated with TP when compared to animals treated with vehicle [F (1, 96) = 70.1, $p < .0001$]. The incidence of apoptosis changed with age [F (5, 96) = 50.3, $p < .0001$], and these changes varied by treatment [F (5, 96) = 19.3, $p < .0001$; Fig. 4(A)]. Post hoc testing revealed that incidence of apoptosis was lower in TP-treated animals than in

vehicle-treated animals on PN 4, PN 6, and PN 8 ($p < .05$). Overall, there was no sex difference in the incidence of apoptosis nor was there an interaction between sex and treatment.

The volume of the BSTpr in animals treated with TP was larger when compared to animals treated with vehicle [F (1, 96) = 52.0, $p < .0001$]. The BSTpr volume increased with age [F (5, 96) = 37.1, $p < .0001$], and this increase varied by treatment [F (5, 96) = 3.5, $p < .01$; Fig. 4(B)]. Post hoc analysis revealed that the volume of the BSTpr was larger in TP-treated animals than in vehicle-treated animals on PN 8, PN 10, and PN 12 ($p < .05$). Overall, there was no sex difference in the volume nor was there an interaction between sex and treatment.

Gonadal Steroid Effects on Apoptosis: MPNc

Because of difficulties in the delineation of the MPNc on PN 2, we analyzed the MPNc only between PN 4 and 12. Of the TP-treated animals three males on PN 4, four males and three females on PN 6, and four males on PN 8 and PN 12 were analyzed. Of the vehicle-treated animals four males and four females on PN 4, four males on PN 6, and four males on PN 10 and 12 were analyzed. Overall, the incidence of apoptosis was significantly reduced in animals treated with TP when compared to animals treated with vehicle [F (1, 69) = 8.7, $p < .005$]. The incidence of apoptosis changed with age [F (4, 69) = 3.9, $p < .01$], and these changes varied by treatment [F (4, 69) = 4.4, $p < .005$; Fig. 4(C)]. Post hoc testing revealed that the incidence of apoptosis was lower in TP-treated animals than in vehicle-treated animals on PN 8 ($p < .05$). Overall, there was no sex difference in the incidence of apoptosis nor was there an interaction between sex and treatment.

The volume of the MPNc was larger in animals treated with TP when compared to animals treated with vehicle [F (1, 69) = 35.6, $p < .0001$]. The MPNc volume significantly changed with age [F (4, 69) = 11.5, $p < .0001$], but these changes varied by treatment [F (4, 69) = 7.25, $p < .0001$; Fig. 4(D)]. Post hoc analysis revealed that the volume of the MPNc was larger in TP-treated animals than in vehicle-treated animals on PN 10 and 12 ($p < .05$). In addition, there was a significant sex difference in MPNc volume [F (1, 69) = 10.1, $p < .005$], but no significant interaction between sex and treatment. Post hoc analysis showed that the MPNc was larger in males than in females on PN 10 ($p < .05$).

Cross-sectional Area of Apoptotic Profiles on PN 6

Overall, there was no sex difference [F (1, 54) = .03, $p = .84$] in the cross-sectional area nor a treatment effect [F (2, 54) = 1.3, $p = .2$] between sham-treated males (27.2 ± 3.7 μm^2), sham-treated females (28.3 ± 3.6 μm^2), TP-treated males (32.0 ± 3.0 μm^2), TP-treated females (30.3 ± 3.1 μm^2), vehicle-treated males (25.2 ± 2.7 μm^2), or vehicle-treated females (27.2 ± 1.7 μm^2). In addition, there was no significant interaction between sex and treatment [F (2, 54) = .2, $p = .82$].

DISCUSSION

In the present study, we found that the incidence of apoptosis in the BSTpr was higher in females than in males, and we also confirmed previous findings that the same was true for the MPNc (Davis et al., 1996). In the BSTl, the incidence of apoptosis was only slightly higher in males than in females, whereas no sex difference in apoptosis was found in the VMH and PVN. Gonadal steroids seem to control the incidence of apoptosis in the BSTpr and MPNc, because TP treatment reduced the incidence of apoptosis in females and castrated males. These results are consistent with the hypothesis that sex differences in perinatal levels of testosterone generate volumetric and numerical sex differences in the BST and MPN, in part, by suppressing naturally occurring apoptosis in males.

In contrast to earlier studies investigating apoptosis during sexual differentiation, terminal deoxynucleotidyl nick-end labeling (TUNEL) (Arai et al., 1996; Davis et al., 1996) was not used to detect apoptotic nuclei. Instead, sections were stained with cresyl violet, which reveals the typical darkly stained and condensed profiles of apoptotic nuclei. Because cresyl violet does not exclusively stain apoptotic cells, some apoptotic nuclei may have been theoretically masked by the cresyl violet staining in nonapoptotic neighboring cells. However, this does not seem to be a major problem, because many studies have shown that the number of apoptotic cells detected with cresyl violet or a similar histological staining strongly correlates with the number of apoptotic cells as visualized by the TUNEL method (e.g., Rabacchi et al., 1994; Bonfanti et al., 1996; Park et al., 1998a).

In the present study, TP was more effective in reducing the incidence of apoptosis in the BSTpr than in the MPNc of females and castrated males. In the BSTpr, the incidence of apoptosis was similar be-

tween sham-treated males from the first analysis and animals treated with TP from the second analysis. The same was true between sham-treated females from the first analysis and animals treated with vehicle from the second analysis. In the MPNc, however, the incidence of apoptosis showed no obvious peak of apoptosis in sham-treated males from the first analysis, whereas a peak of apoptosis was observed around PN 10 in animals treated with TP in the second analysis. These MPNc results seem to be inconsistent with earlier findings that showed that TP completely inhibited developmental apoptosis in the MPNc (Davis et al., 1996). However, this disparity may well be explained by differences in the timing of TP treatment. In earlier studies, pups received TP several days after birth (Davis et al., 1996), whereas in the present study TP was given on the day of birth, which may have resulted in different TP levels during the MPNc cell death period. Differences in the pattern of ontogenesis between BSTpr and MPNc cells may be responsible for the difference in effectiveness of TP in curtailing the incidence of apoptosis in these two areas. Counting the day of conception as embryonic day 1, BSTpr cells are born around embryonic day 16 and 17, whereas MPNc cells are born 1 to 2 days later (Jacobson et al., 1981b; Bayer, 1987; Bayer and Altman, 1987). Hence, perinatal BSTpr cells may well be more advanced in their development than MPNc cells during the same perinatal period. An indication for such a difference may be the level of androgen receptor (AR) mRNA expression, which is present in high abundance in the perinatal BSTpr and MPN (McAbee and DonCarlos, 1998). Indeed, the perinatal AR mRNA expression between the BSTpr and MPN appears to develop more rapidly in the BSTpr than in the MPN (McAbee and DonCarlos, 1998), supporting the idea that neonatal TP administration may protect the more mature BSTpr cells better against postnatal apoptosis than the more immature MPNc cells.

Our findings in the VMH and BSTl demonstrate that the presence or absence of sex differences in apoptosis does not necessarily predict the presence or absence of sex differences in volume. For example, although the VMH showed no sex difference in the incidence of apoptosis, its volume was larger in males than in females on PN 12. The sex difference in VMH volume is in agreement with earlier findings suggesting that the rat VMH is larger in males than in females (Matsumoto and Arai, 1983). This volumetric sex difference is likely to be a function of the perinatal sex difference in gonadal steroids, because manipulation of gonadal steroid level affected the VMH volume (Matsumoto and Arai, 1983). It is unknown how gonadal steroids promote the sexual differentiation of the VMH. In the absence of a sex difference of apoptosis, it may be that gonadal steroids act on soma size, as they appear to do in the preoptic area of the ferret brain (Park et al., 1998a,b). Therefore, we propose that the volumetric sex difference found in the VMH may be a result of gonadal steroid action on the cell soma size, indicating that differentiation of cell phenotype may be important for the sexual differentiation of the VMH.

It is unknown precisely where gonadal steroids act to influence apoptosis during sexual differentiation. In case of the BSTpr, gonadal steroids may act directly on BSTpr cells to prevent apoptotic cell death. Indeed, mRNA for gonadal steroid receptors is highly concentrated in neonatal BSTpr cells (DonCarlos and Handa, 1994; McAbee and DonCarlos, 1998). Alternatively, gonadal steroids may promote BSTpr cell survival indirectly by preventing apoptosis in important projection areas, such as the MPN or AVPv (Simerly and Swanson, 1986; Hutton et al., 1998), which also contain high levels of androgen receptor and estrogen receptor-α mRNA (DonCarlos and Handa, 1994; McAbee and DonCarlos, 1998). Indeed, the present and earlier studies showed that gonadal steroids regulate apoptosis in the MPN and AVPv (Arai et al., 1996; Davis et al., 1996). Such an indirect mechanism underlies the sexual differentiation of the motoneurons in the SNB in the rat spinal cord. Testosterone promotes the survival of cells in perineal muscles that are innervated by the SNB motoneurons. In the absence of testosterone, these muscle cells die (Cihak et al.,1970) and, consequently, SNB motoneurons die as well (Nordeen et al., 1985). Moreover, testosterone cannot prevent SNB motoneurons cell death in the absence of the perineal muscles (Kurz et al., 1992). Presumably, the target muscle cells provide trophic factors such as ciliary neurotrophic factor, which has been shown to prevent SNB motoneurons from dying (Forger et al., 1993).

In analogy, gonadal steroid action on the MPN and AVPv may affect apoptotic cell death in the BSTpr in a similar fashion. However, it is unlikely that testosterone prevented apoptosis in the BSTpr by acting on AVPv and/or MPN cells, because BSTpr fibers projecting to the AVPv and MPN seem to be established after the apoptosis period in the BSTpr, in fact, between PN 8 and PN 10 (Hutton et al., 1998). Furthermore, in contrast to the SNB motoneurons, BSTpr cells express receptors for gonadal steroids during development (DonCarlos and Handa, 1994; McAbee and DonCarlos, 1998). Therefore, it is more likely that gonadal steroids regulate the incidence of apoptosis directly in the BST. Because of the robust effects of gonadal steroids on apoptosis in the BST,

this area appears well-suited to investigate the molecular and cellular mechanisms that regulate sex-dependent apoptosis.

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