

Development of Sex Differences in the Principal Nucleus of the Bed Nucleus of the Stria Terminalis of Mice: Role of *Bax*-Dependent Cell Death

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ABSTRACT: Neuron number in the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) is greater in adult male mice than in females. Deletion of the proapoptotic gene, *Bax*, increases the number of BNSTp cells in adulthood and eliminates the sex difference in cell number. Here, we map the ontogeny of sex differences in nuclear volume and cell number in the BNSTp of neonatal mice, and evaluate the role of cell death in the development of these differences. We find that BNSTp volume and cell number do not differ between male and female wild-type mice on postnatal days P3, P5, or P7. Sex differences emerge after the first postnatal week and both measures are significantly greater in males than in females on P9 and P11. Cell death, assessed by TUNEL staining, was observed in the

BNSTp of both sexes from P1–P8. Females had more TUNEL-positive cells than males from approximately P3–P6, with the maximum number of dying cells observed on P5/P6. To test whether the *Bax* gene is required for sexually dimorphic cell death in the BNSTp, TUNEL cells were counted on P6 in *Bax*^{-/-} mice and their *Bax*^{+/+} siblings. *Bax* gene deletion nearly abolished TUNEL-positive cells in the BNSTp of both sexes. Together, these findings support the interpretation that the sex difference in BNSTp cell number seen in adulthood is due to *Bax*-dependent, sexually dimorphic cell death during the first week of life.

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INTRODUCTION

Several of the best studied neural sex differences are differences in cell number (Forger, 2006 for review). For example, male rats have more neurons than do females in the spinal nucleus of the bulbocavernosus (SNB) and the sexually dimorphic nucleus of the preoptic area (SDN-POA), whereas females have more cells than do males in the anteroventral periventricular nucleus (AVPV; Breedlove and Arnold, 1980;

Dodson and Gorski, 1993; Sumida et al., 1993). Differential cell death in males and females during perinatal life has been implicated as the basis for these sex differences, based on inverse correlations between neuron number in adulthood and the number of dying cells during development (Nordeen et al., 1985; Sumida et al., 1993; Davis et al., 1996).

More recently, the principal nucleus of the bed nucleus of the stria terminalis (BNSTp, also known as the “encapsulated” region of the BNST, or the medial posterior BNST) has been added to the list of structures likely to be differentiated by hormonally regulated cell death. The BNSTp is an important component of the sexually dimorphic circuitry integrating endocrine and environmental signals in the control of reproductive function (reviewed in De Vries and Sim-

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erly, 2002). BNSTp volume and cell number are greater in male than in female rats in adulthood (del Abril et al., 1987; Hines et al., 1992), and these sex differences are eliminated by castrating males or treating females with testosterone on the day of birth (Guillamon et al., 1988). Although cell counts in the developing BNSTp of rats have not been reported, the sex difference in nuclear volume emerges between postnatal days 10 (P10) and 12 (Chung et al., 2000). Female rats have more pyknotic cells in the BNSTp than do males on P6, and manipulating gonadal steroids in newborns eliminates both the sex difference in cell death and the later-emerging difference in volume (Chung et al., 2000). Thus, as in the SNB, SDN-POA, and AVPV (Nordeen et al., 1985; Murakami and Arai, 1989; Davis et al., 1996), hormonally regulated cell death during neonatal life likely underlies the sexual differentiation of cell number in the BNSTp of rats. However, the molecular and cellular mechanisms whereby steroids control developmental cell death are not known for the BNSTp, or for any other cell group.

The large number of transgenic and knockout mouse strains currently available may permit a genetic dissection of the molecular pathways controlling hormone-regulated, sexually dimorphic cell death. We recently found that BNSTp volume and cell number is sexually dimorphic in adult C57BL/6 mice as it is in rats, and this sex difference is eliminated in mice with a targeted deletion of the *Bax* gene (Forger et al., 2004). *Bax* is a member of the Bcl-2 family of proteins, which controls death in many cell types (Merry and Korsmeyer, 1997). Some family members promote cell survival (e.g. Bcl-2 itself) whereas others promote death. *Bax* is the best characterized of the death-promoting family members; deletion of this gene eliminates cell death in many neural regions during development (White et al., 1998), as well as in adulthood (Sun et al., 2004).

We proposed that the deletion of *Bax* eliminated the sex difference in cell number in the BNSTp of adult mice by preventing sexually dimorphic cell death during development (Forger et al., 2004). The current study was designed to test this hypothesis and to pinpoint the timing of *Bax*-dependent cell death in the BNSTp, as a critical first step in identifying the molecular bases of hormone-regulated cell death. Specifically, this study addressed three goals: (1) to determine when the BNSTp of wild-type mice becomes sexually dimorphic in volume and cell number; (2) to map the timing of developmental cell death in the BNSTp of males and females; and (3) to examine the effect of *Bax* gene deletion on cell death in the BNSTp of neonatal mice.

METHODS

Animals

Mice were housed in a 12:12 light/dark cycle and held at 22°C. The normal ontogeny of BNSTp volume and cell number was determined using pups from our wild-type C57BL/6 breeding colony at the University of Massachusetts; an additional cohort of animals from this colony was used for analysis of cell death by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling). *Bax* knockout (*Bax*^{-/-}) pups and their wild-type (*Bax*^{+/+}) siblings were obtained by mating males and females heterozygous for the *Bax* gene deletion (The Jackson Laboratory, Bar Harbor, ME). These animals were originally generated in a mixed C57Bl/6 × 129 background (Knudson et al., 1995), and subsequently were backcrossed to pure C57Bl/6 for eight generations. Offspring were genotyped by PCR amplification of tail DNA using published primer sequences (White et al., 1998). All of the measurements described below were performed on slides coded to conceal the sex, age, and genotype of the animals.

BNSTp Volume and Cell Counts

Volume and cell number of the BNSTp were determined between P3 and P11 (day of birth = P1). Pups were killed by rapid decapitation on P3 (3 male, 5 female), P5 (6 male, 5 female), P7 (6 male, 6 female), P9 (4 male, 6 female), and P11 (5 male, 5 female); no more than two pups (one of each sex) from a single litter contributed to each time point. Brains were removed from the skull and immersion fixed in Bouin's solution, embedded in paraffin, serially sectioned at 15 μm, and stained with thionin. Nuclear volume measurements and stereological cell counts were performed as previously (Forger et al., 2004). Briefly, StereoInvestigator software (MicroBrightfield, Williston, VT) was used to trace the outline of the BNSTp unilaterally in every fourth section. Volume was estimated by multiplying the summed cross-sectional areas by 60 μm (the distance between traced sections). The optical disector method was used for cell counts, by moving a 20 by 20 μm² counting frame through the traced contours of the BNSTp. Cells were counted that exhibited the morphological characteristics of neurons and had a clearly visible nucleus that came into focus within the counting frame. Upper and lower guard zones of 1 μm were used to avoid edge artifacts, and the spacing between counting frames was 10,000 μm² (a sampling grid of 100 × 100 μm).

TUNEL

The normal pattern of cell death in the BNSTp was determined by counting TUNEL-positive cells in wild-type mice between P1 and P8. Because the number of mice sacrificed on any single day for this analysis was small, animals were combined to form four groups as follows: P1 or P2 (P1/P2; 6 males, 6 females); P3/P4 (7 male, 4 female); P5/P6

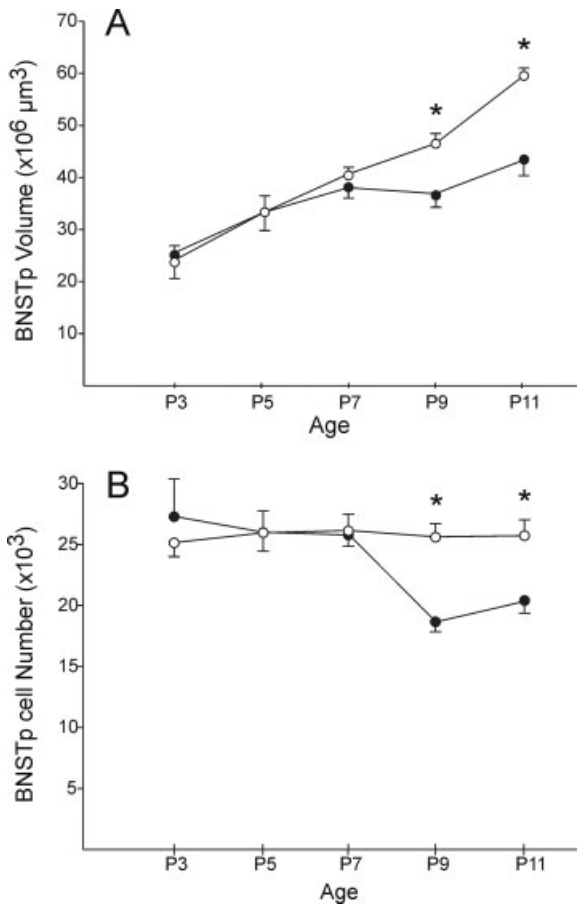


Figure 1 Development of sex differences in volume (A) and cell number (B) in the BNSTp of perinatal mice. Both measures are similar in males (○) and females (●) during the first postnatal week, but are greater in males on postnatal days 9 and 11.

(5 male, 6 female); and P7/P8 (3 male, 5 female). In addition, several *Bax*^{-/-} mice (4 male, 4 female) and their *Bax*^{+/+} siblings (2 male, 3 female) were examined on P6. Mice were anesthetized with ketamine and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 10 days, embedded in paraffin, and cut at 15 μm. Every third section was processed for the detection of DNA fragmentation using the Apoptag kit (Chemicon International, Temecula, CA), following recommendations of the manufacturer. Sections were counter-stained with methyl green. The BNSTp was traced unilaterally throughout its extent with the aid of StereoInvestigator software and TUNEL-positive cells were counted using the “meander scan” function of StereoInvestigator to systematically move through the BNSTp. Counts were multiplied by three to correct for sampling ratio.

Data Analysis

Two-way ANOVAs (sex-by-age) were used to analyze volume, overall cell number, and the number of TUNEL-

labeled cells in the BNSTp of wild-type mice. The effects of *Bax* gene deletion on developmental cell death were evaluated using a 2-way, sex-by-genotype ANOVA. Pairwise comparisons were performed using Fisher’s LSD.

RESULTS

Development of a Sex Difference in BNSTp Volume and Cell Number

Two-way ANOVA revealed a main effect of age ($F_{4,41} = 22.3$; $p < 0.0005$), a main effect of sex ($F_{1,41} = 9.0$; $p = 0.005$), and a sex-by-age interaction ($F_{4,41} = 3.4$; $p < 0.02$) on BNSTp volume [Fig. 1(A)]. Volumes increased significantly with age and were greater in males. This sex difference emerged after the first postnatal week: there were no sex differences in BNSTp volume on P3, P5, or P7, but volumes were significantly greater in males than in females on P9 and P11 ($p < 0.02$ and $p < 0.0005$, respectively).

A similar time course was observed for the development of a sex difference in BNSTp cell number [Fig. 1(B)]. Stereological cell counts did not differ between males and females on P3, P5, or P7, but males had a greater number of cells in the BNSTp than did females on P9 and P11 ($p < 0.01$ and $p < 0.03$, respectively). The emergence of this sex difference was due to a decrease in cell number in females: cell counts were relatively constant in males of all ages studied, but declined by 28% in females between P7 and P9 [$p < 0.005$; Fig. 1(B)]. These findings were reflected in a main effect of age ($F_{4,41} = 2.6$; $p < 0.05$), a marginally significant effect of sex ($F_{1,41} = 3.9$; $p = 0.055$), and a marginally significant sex-by-age interaction ($F_{4,41} = 2.5$; $p = 0.055$) on cell number in the 2-way ANOVA.

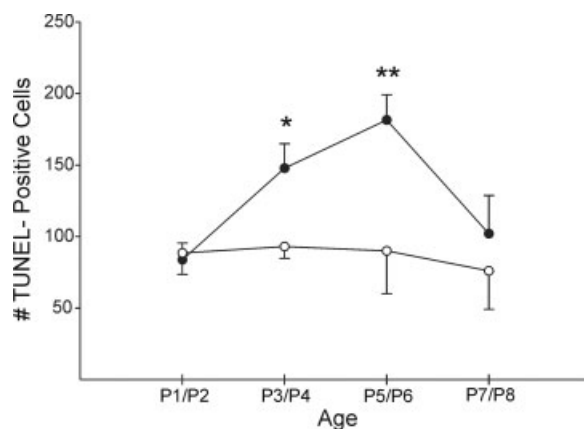


Figure 2 Counts of TUNEL-positive cells in the BNSTp of neonatal male (○) and female (●) wild-type mice. Cell death peaks on P5/P6 in females and is greater in females than in males on P3/P4 and P5/P6.

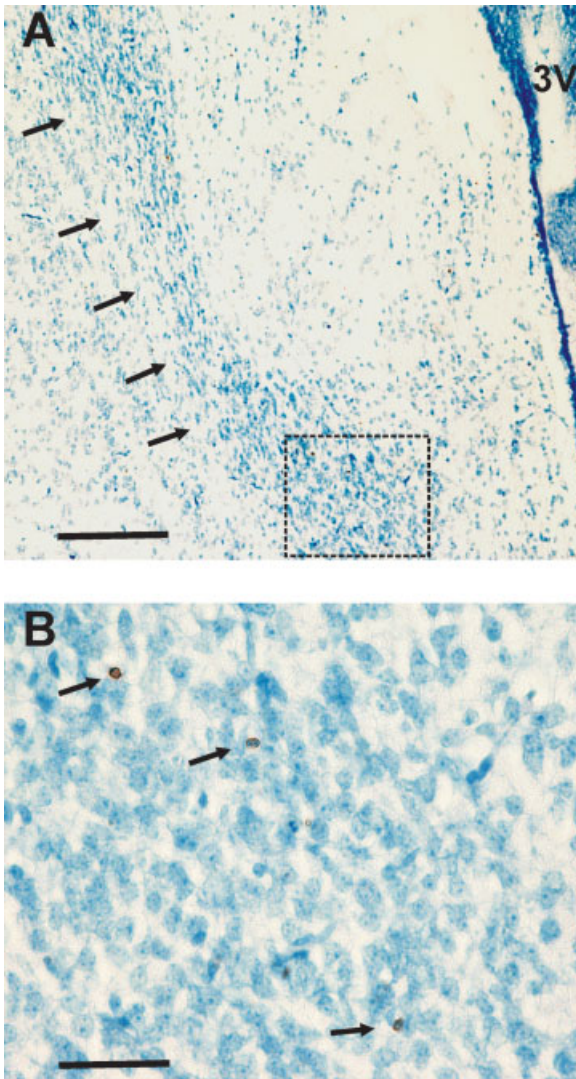


Figure 3 (A) Low-power magnification of the BNSTp (arrows) of a male wild-type mouse on P4; 3V, third ventricle. Scale bar = 200 μm . (B) Higher power view of the ventral extension of the BNSTp [boxed area in (A)]. Arrows point to three TUNEL-positive cells. Scale bar = 50 μm .

Time Course of Apoptosis in the BNSTp

Counts of TUNEL-positive cells indicate main effects of both sex ($F_{1,34} = 9.3$; $p < 0.01$) and age ($F_{3,34} = 3.3$; $p < 0.05$) on cell death in the BNSTp of C57Bl/6 mice (Figs. 2 and 3). Overall, the number of dying cells was greater in females than in males. This was due to marginally higher TUNEL counts in females on P3/P4 ($p = 0.05$) and significantly greater numbers of dying cells in females than in males on P5/P6 ($p < 0.01$). Although TUNEL-labeled cells were found in both sexes at all ages, the peak number of dying cells in the mouse BNSTp was found on P5/P6.

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Effect of *Bax* on Cell Death in the BNSTp

To test whether *Bax* is required for sexually dimorphic cell death in the neonatal BNSTp, we counted TUNEL-positive cells in *Bax* $-/-$ mice and their *Bax* $+/+$ littermates on P6, the peak of cell death as determined above for wild-type mice. Two-way ANOVA indicates a main effect of sex ($F_{1,9} = 8.3$; $p < 0.02$), a main effect of genotype ($F_{1,9} = 81.0$; $p < 0.0005$), and a sex-by-genotype interaction ($F_{1,9} = 6.6$; $p < 0.05$) on the number of TUNEL-positive cells. Female *Bax* $+/+$ mice had significantly more apoptotic cells in the BNSTp than did *Bax* $+/+$ males ($p < 0.01$; Fig. 4), confirming the sex difference observed in wild-type C57Bl/6 mice. The absolute number of dying cells in the BNSTp of male and female *Bax* $+/+$ mice on P6 also was similar to that seen on P5/P6 in mice from our wild-type C57Bl/6 colony (compare Figs. 2 and 4).

Cell death was severely reduced in *Bax* $-/-$ mice of both sexes ($p < 0.0005$ and $p < 0.01$ in males and females, respectively), and a Fisher's post-hoc test indicates that the sex difference in TUNEL-positive cells was eliminated in *Bax* $-/-$ mice ($p > 0.5$; Figs. 4 and 5). However, cell death in the BNSTp was not completely abolished in *Bax* $-/-$ mice. We counted between one and three TUNEL-positive cells in each *Bax* $-/-$ female and a single TUNEL-positive cell in one of the four *Bax* $-/-$ males. This leaves open the possibility that a small amount of *Bax*-independent cell death occurs in the BNSTp, and could contribute to the sex difference in cell number.

Although we did not quantify cell death in areas outside of the BNSTp, it was evident that *Bax* deletion markedly reduced the number of TUNEL-positive

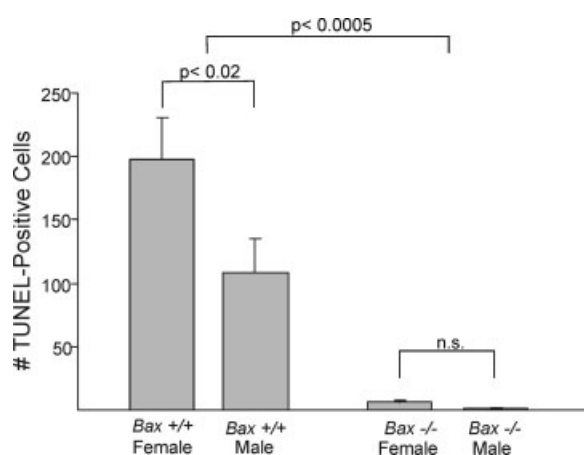
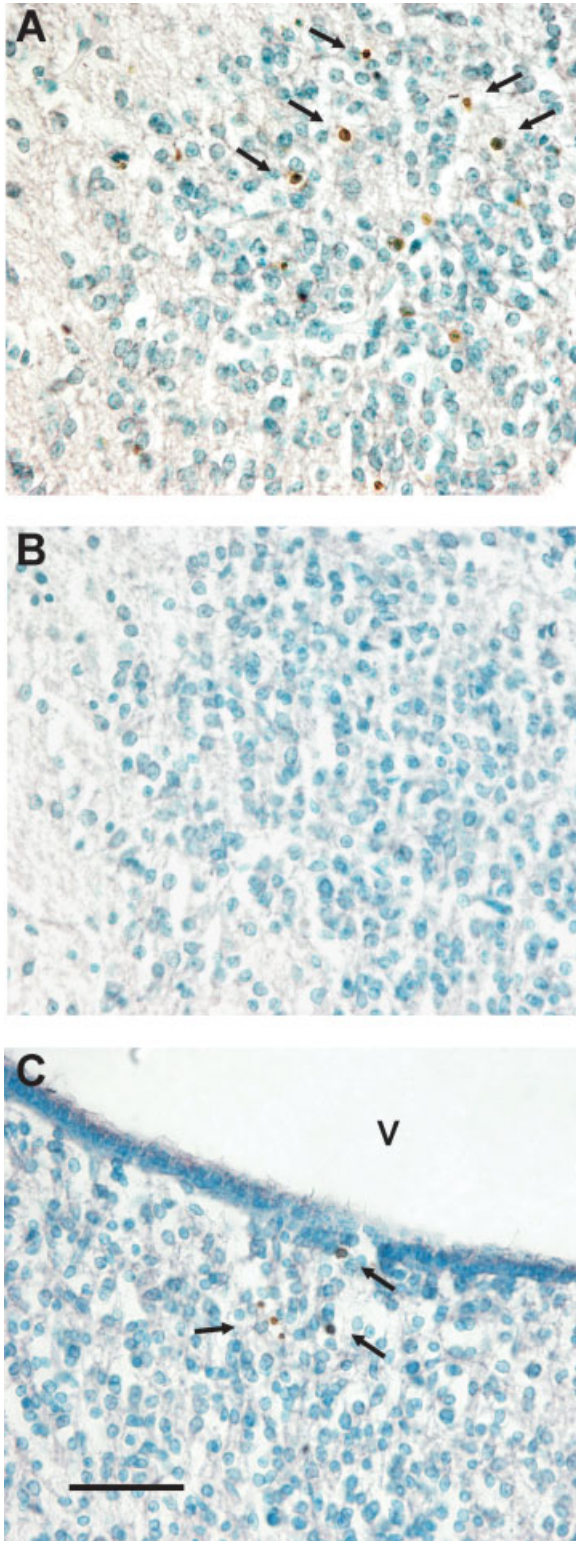


Figure 4 Counts of TUNEL-positive cells in the BNSTp of *Bax* $+/+$ and *Bax* $-/-$ mice on P6. *Bax* $+/+$ females have more dying cells than do *Bax* $+/+$ males. Cell death in the BNSTp is nearly eliminated in *Bax* $-/-$ mice of both sexes.

tive cells in many brain regions, as reported previously (Shindler et al., 1997; White et al., 1998). All brain areas were not equally affected, however. For example, TUNEL-positive cells could often be found



just dorsal to the BNSTp, in a region abutting the ventral-most extension of the lateral ventricle (Fig. 5) and in fiber bundles such as the corpus callosum and anterior commissure (not shown). These observations are consistent with the suggestion that *Bax* is not required for the death of non-neuronal cells or for apoptosis of neural precursor cells (White et al., 1998; D'Sa-Eipper et al., 2001).

DISCUSSION

The BNST is an important node in a sexually dimorphic circuit controlling reproductive function, and a site of abundant androgen and estrogen receptor expression throughout life (Simerly et al., 1990; Sibug et al., 1991; Handa et al., 1996; Shughrue et al., 1997; McAbee and DonCarlos, 1998; Mitra et al., 2003). Sexual stimuli induce Fos immunoreactivity in the BNST of males in several species (Baum and Everitt, 1992; Wood and Newman, 1993; Baum et al., 1994; Kippin et al., 2003), and lesions to this region disrupt male sexual behavior (Emory and Sachs, 1976; Claro et al., 1995). The BNST is also involved in control of the luteinizing hormone surge in females (Beltramino and Taleisnik, 1980), and a strong, sex-specific inhibitory input from the BNSTp to the AVPV develops during the second postnatal week that may contribute to the inability of male rats to display female-typical surges (Hutton et al., 1998; Gu et al., 2003; Polston et al., 2004). Thus, sexual dimorphism of the BNSTp may underlie sex differences in behavior and/or neuroendocrine function.

The data presented here demonstrate that a sex difference in BNSTp cell number first emerges on P9 in mice and results from a decrease in the number of cells in females. TUNEL-positive cells are present in the BNSTp of both sexes from P1–P8, and are more numerous in females on P3/P4 and P5/P6. A similar sex difference in apoptosis is seen in *Bax* $+/+$ animals on P6, and a null mutation of the *Bax* gene nearly eliminates TUNEL-positive cells in the BNSTp of both males and females. Taken together, the sex difference in BNSTp cell number seen previously in adult mice

Figure 5 (A) Photomicrograph through the dorsal BNSTp of a *Bax* $+/+$ female on P6. Arrows point to several of the many TUNEL-positive profiles in this view. (B) Photomicrograph through the dorsal BNSTp of a *Bax* $-/-$ female on P6. No TUNEL-positive profiles are seen. (C) TUNEL-positive cells near the lateral ventricle (V) of a *Bax* $-/-$ female. View shown in (C) is from the same section as (B), $\sim 300 \mu\text{m}$ medial to the BNSTp. Medial is to the right for all views. Scale bar = $50 \mu\text{m}$.

(Forger et al., 2004) can be attributed, at least in part, to differential *Bax*-dependent cell death in males and females during neonatal life. As far as we are aware, cell counts have not been reported for the developing BNSTp of any other species. However, a similar time-course is observed for the emergence of a sex difference in BNSTp volume in rats: female rats have more pyknotic cell profiles in the BNSTp on P6 than do males, and a sex difference in volume (male > female) emerges several days later, (between P10 and P12; Chung et al., 2000).

These findings are in accordance with a literature ascribing sex differences in cell number in several regions of the nervous system to hormone-regulated, developmental cell death (Nordeen et al., 1985; Murakami and Arai, 1989; Davis et al., 1996; Nuñez et al., 2000). A closer look at our data and those of others, however, raises some questions. For example, cell counts in males remained essentially constant between P3 and P11, despite the fact that a substantial number of TUNEL-positive cells are observed in the BNSTp of both sexes on each day examined (P1–P8). It is possible that some TUNEL-labeled cells might be an artifact of the staining procedure (i.e., false positives), but this seems unlikely to account for our data: TUNEL-positive cells in the BNSTp were virtually absent in *Bax* $-/-$ mice, and artifacts of the labeling technique would be expected regardless of genotype. We suggest instead that cell recruitment and cell death overlap in the developing BNSTp of both sexes, and that the migration of cells into the BNSTp during perinatal life balances out developmental cell death in males. Similarly, during development of other sexually dimorphic nuclei such as the SNB, SDN-POA, and locus coeruleus of rats, the period of naturally occurring cell death overlaps with neurogenesis and/or cell migration (Jacobson and Gorski, 1981; Nordeen et al., 1985; Davis et al., 1996; Pinos et al., 2001). In fact, despite substantial numbers of apoptotic cells throughout the SDN-POA of both sexes during the first two postnatal weeks, total cell number actually increases in male rats during this interval (Davis et al., 1996).

Neurons destined for the BNSTp are generated later than most surrounding cells, with the majority undergoing their final division between embryonic day (E) 14 and E17 in the rat (Bayer, 1987; Bayer and Altman, 1987; Han and De Vries, 1999) and E12 to E15 in the mouse (Crepes, 1974). At least some of the cells born on E17 in rats have reached their final destination in the BNSTp by E21/E22 (Bayer, 1987). If migration into the nucleus continues into the second postnatal week, as we suggest, then either a sub-population of BNSTp cells migrates very slowly,

and/or the aggregation of cells to form clear nuclear boundaries occurs only gradually. Similarly, although some of the SDN-POA neurons born on E18 in rats reach their final destination by P1, the majority do not congregate in the nucleus until much later (P6–P10; Jacobson et al., 1985). The signals that determine the postnatal aggregation of cells into identifiable nuclei are largely unknown. If such signals are modified by gonadal steroid hormones, then sex differences in migration and aggregation may contribute to transient (i.e., observed only during development) or permanent morphological neural sex differences (Wolfe et al., 2005). It also remains possible that there is a previously unrecognized population of late-born cells in the BNST and elsewhere. Recently, a small number of cells expressing markers of migrating neuroblasts were unexpectedly found in the dorsal BNST and adjacent regions of adult mice (Yang et al., 2004).

Another curious feature of our data is the observation that cell death is significantly greater in females than in males from approximately P3–P6, yet sex differences in volume and cell number do not emerge until after P7. A delay between the appearance of a sex difference in cell death and a volume sex difference is also seen in the rat BNSTp (Chung et al., 2000) and could be due to the growth and/or redistribution of cell bodies after the cell death period. The delayed emergence of a sex difference in cell number is more difficult to explain. It is possible that cells in the early stages of DNA fragmentation may be both TUNEL-positive and countable as neurons in a Nissl-stain. If so, and if the process of apoptosis is relatively protracted, there might be a lag between an increase in TUNEL-positive cells and a decrease in overall cell number.

The length of time a dying cell remains identifiable before being cleared by phagocytosis is not known for the BNSTp or most other tissues. This variable is difficult to measure, and attempts to calculate it have generated estimates ranging from <1 h to over 48 h (Bursch et al., 1990; Voyvodic et al., 1995; Hu et al., 1997; Thomaidou et al., 1997; Nuñez et al., 2001). The data presented here allow us to make a rough calculation of how long a dying cell remains detectable by TUNEL-labeling in the developing BNSTp, if certain assumptions are granted. For example, if the sex difference in BNSTp cell number on P9 and P11 can be accounted for by differences in the number of TUNEL-positive cells between P3 and P8 in males and females, then the approximately 6000 more cells in males must be accounted for by ~390 “extra” TUNEL cells in the BNSTp of females (~70 more apoptotic cells in females on each of days P3 and P4, ~100 more on P5 and P6, and ~25 more

on P7 and P8). For this to be true, each dying cell must appear TUNEL-positive for ~ 1.6 h [specifically: $390 \text{ cells}/(x/24 \text{ h}) = 6000 \text{ cells}$; $x = 1.56 \text{ h}$].

There are several caveats associated with a post-hoc calculation such as this, however. One must assume that the amount of cell death and the clearance rate of dying cells are relatively constant throughout the circadian day, that clearance rates do not vary at different postnatal ages, and that the length of time a dying cell remains detectable does not vary between the sexes. These assumptions have not been tested for the BNSTp or other sexually dimorphic brain regions; if the last assumption is not correct, then all sex differences in cell death based on counts of dying cells would be subject to reinterpretation.

At present, however, we tentatively conclude that a protracted process of apoptosis during which cells within the BNSTp may be both TUNEL-positive and counted as neurons in a Nissl stain is unlikely to account for the lag between observed sex differences in cell death and a sex difference in total cell number. Although we believe that the evidence is quite strong that developmental cell death contributes importantly to the sex difference in overall BNSTp cell number, other processes such as migration, and/or the aggregation and condensation of cells to form recognizable nuclear boundaries may also be involved. In addition, the BNSTp is a large and heterogeneous nucleus in which sex differences in several neurochemically defined cell types have been described (van Leeuwen et al., 1985; Micevych et al., 1988; Wersinger et al., 1997). It is possible, perhaps even likely, that subpopulations of cells within the nucleus may be differentially affected by sex differences in cell death, migration, aggregation, and/or the hormonal specification of neurotransmitter/neuropeptide expression. Parsing this out in the BNSTp and other sexually dimorphic cell groups is the next challenge and an important step in attaining a more sophisticated understanding of the development of sex differences in cell number.

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