

Neurogenesis of Galanin Cells in the Bed Nucleus of the Stria Terminalis and Centromedial Amygdala in Rats: A Model for Sexual Differentiation of Neuronal Phenotype

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ABSTRACT: Male rats possess twice as many cells that express arginine-vasopressin (AVP) in the bed nucleus of the stria terminalis (BST) and centromedial amygdala (CMA) as do females. This sex difference may arise from sex differences in the induction of AVP expression in galanin (GAL)-expressing cells, which themselves do not differ in number between males and females. To test whether AVP expression could arise from a single pool of galaninergic cells, we determined whether the cell birth profile of GAL-immunoreactive (ir) cells was similar to that of AVP-ir cells. Dams were injected with the cell birth marker bromodeoxyuridine (BrdU) on one of seven gestational dates, ranging from embryonic day 11 (E11) to E17. The resulting offspring

were sacrificed at 3 months of age. Processing their brains for the presence of either GAL and BrdU, or AVP and BrdU immunoreactivity revealed that in both the BST and CMA, the majority of GAL-ir and AVP-ir cells were labeled with BrdU on E12 and E13. In contrast, most other cells in the same region were labeled on E14 and E15. The similarity in the timing of cell birth of the GAL-ir and AVP-ir cells is consistent with the idea that GAL-ir cells in the BST/CMA constitute a single pool of cells that may be induced to express AVP during development. © 1999 John Wiley & Sons, Inc. *J Neurobiol* 38: 491–498, 1999

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During a restricted period in development, sex differences in gonadal steroid levels induce permanent differences in brain structure and function (Breedlove, 1992). Hormones have been shown to induce differences in cell number and phenotype in certain areas (Kawata, 1995). Hormones may influence cell number by influencing programmed cell death, which appears

to be under hormonal control in sexually dimorphic areas (Murakami and Arai, 1989; Davis et al., 1996; Arai et al., 1996; Nordeen et al., 1985). Hormones may also influence cell number through processes such as neurogenesis and cell migration (Tobet and Hanna, 1997). Hormones determine neuronal phenotype by influencing parameters such as dendritic arborization and neurite outgrowth (Greenough et al., 1977; Toran-Allerand, 1980; Simerly, 1998) and the expression of neurotransmitters and their receptors (De Vries, 1990; McCarthy, 1997). The molecular and cellular bases of hormonal effects on neuronal phenotype are largely unknown, partly because of a

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lack of markers for differentiating cells. Because the sexually dimorphic arginine-vasopressin (AVP) system of the brain is neurochemically distinct, studies on its development may reveal molecular steps involved in steroid-dependent differentiation of neuronal phenotype.

Male rats possess two to three times the number of AVP-expressing cell bodies in the bed nucleus of the stria terminalis (BST) and centromedial amygdala (CMA), as well as two to three times higher density of AVP-immunoreactive (ir) projections to the lateral septum as do females (De Vries et al., 1981; Van Leeuwen et al., 1985; Miller et al., 1989). These differences depend, at least in part, on differences in gonadal steroid hormone levels in development (Wang et al., 1993). For example, a sex difference in the number of AVP-ir cells in the BST/CMA could result from differences in the development of neurotransmitter phenotype of individual cells or from differences in neuronal cell death. *In situ* hybridization studies conducted by Miller and colleagues (1993b) found that virtually all AVP mRNA-expressing cells in the BST/CMA also express GAL mRNA. However, not all galanin (GAL) cells express AVP mRNA, nor are they sexually dimorphic in number. Rather, they are sexually dimorphic with respect to AVP coexpression; more of these GAL cells coexpress AVP mRNA in males than in females (Miller et al., 1993b; Planas et al., 1995). To determine whether all galanergic cells in the BST and CMA could represent a single set of cells that are differentially committed during development to express AVP, we conducted a birthdating study using the cell birth marker bromo-2-deoxy-5-uridine (BrdU). If the sexually dimorphic expression of AVP does arise from a subset of GAL cells, then the profile of cell birth of GAL-ir cells should match that of AVP-ir cells.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley rats (Taconic Labs, Germantown, NY) were paired in hanging cages in a 14:10 h light/dark cycle. The day a sperm plug was found was designated embryonic day 1 (E1). Inseminated females were removed and singly housed in plastic tubs. Each dam was given one injection of BrdU (50 mg/kg; Sigma) dissolved in 7 mM sodium hydroxide and 0.9% saline on 1 of 7 days during gestation, from E11 to E17. At least three females were injected for each time point of

the study. Injections were delivered intraperitoneally (i.p.) between 2 and 3 p.m.

Birth occurred on E22–23 and litters were culled to four males and four females by postnatal day 2 (P2). Pups were weaned on P25 and group-housed in hanging cages. At P90, they were given a lethal injection of chloral hydrate/pentobarbital (containing 0.25 M chloral hydrate, 0.08 M magnesium sulfate, 4.5 mM pentobarbital, 2.4 M ethyl alcohol, and 4.7 M propylene glycol; 2.8 mL/kg body weight, i.p.) and intracardially perfused with 0.9% saline followed by 5% acrolein in 0.1 M sodium phosphate buffer (pH 7.6). Fixed brains were then placed in a solution of 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.6) and stored at 4°C. Brains were sectioned into a series of four 40- μ m transverse sections such that one series contained sections 160 μ m apart.

Immunocytochemistry

Free-floating sections were rinsed with Tris-buffered saline (TBS) (0.05 M Trizma base, 0.9% NaCl, pH 7.6) and treated with the following solutions at room temperature: (a) 1% sodium borohydride for 15 min; (b) TBS for 3 \times 5 min; (c) 2N hydrochloric acid for 30 min; (d) TBS for 2 \times 5 min; (e) 20% normal goat serum (NGS) and 0.3% Triton-X (Sigma) in TBS (Tris-Triton) for 15 min; (f) mouse anti-BrdU monoclonal antiserum (Becton-Dickinson, San Jose, CA), diluted 1:200 in 2% NGS in Tris-Triton overnight; (g) 2% NGS in Tris-Triton for 3 \times 5 min; (h) biotinylated goat anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA), diluted 1:200 in 2% NGS in Tris-Triton for 1 h; (i) Tris-Triton for 2 \times 5 min; (j) TBS for 5 min; (k) avidin-biotin peroxidase complex (ABC, Vector Elite Series, Vector) in TBS for 1 h; and (l) TBS 3 \times 5 min. The sections were then incubated in a solution of 0.4% ammonium chloride, 0.75 mM nickel ammonium sulfate, 0.05% diaminobenzidine (DAB), 0.001% glucose oxidase (Type VII-S; Sigma), and 0.3% β -D-glucose to produce a black reaction product (Záborsky and Heimer, 1989). Following the reaction, sections were washed three times with TBS and stored at 4°C.

The next morning, sections were incubated as indicated in Steps f–l with the following exceptions: (a) The primary antibody used was either anti-galanin (1:2000; Peninsula Labs, Belmont, CA) for one series of sections or anti-arginine-vasopressin (1:2000; ICN, Lisle, IL) for another series of sections. Both antisera were diluted in 2% NGS in Tris-Triton and the incubation was run for 2.5 h at 37°C. (b) The secondary antibody used was biotinylated goat anti-rabbit immunoglobulin G, diluted 1:300 in 2% NGS in Tris-Triton. The sections were then stained with a conventional DAB reaction (0.05% DAB and 0.0003% H₂O₂) to produce a brown reaction product. Preadsorbing the GAL and AVP antiserum with 10 mM GAL (Peninsula Labs) or 50 mM AVP (Sigma), respectively, blocked immunostaining. Sections were mounted on slides, air-dried, coverslipped with Permount, and coded.

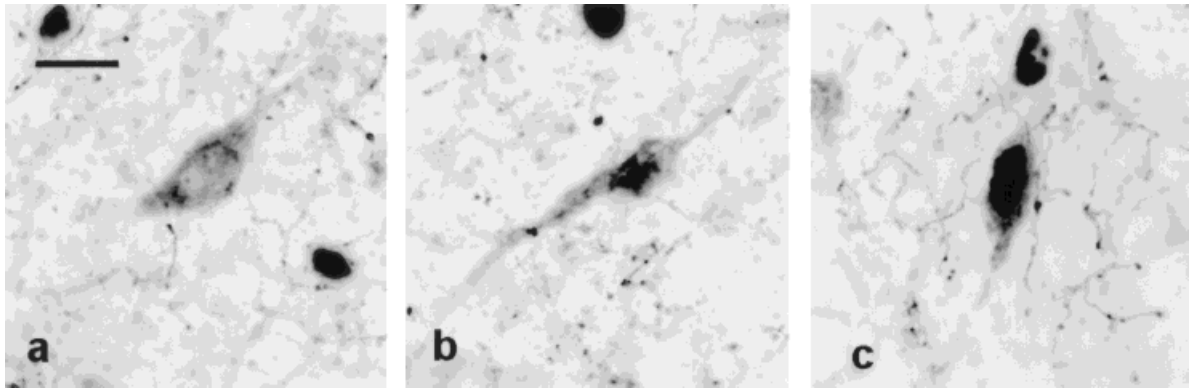


Figure 1 Degrees of BrdU-labeling in GAL-ir cell bodies: (a) none, (b) light, (c) dark. The cells shown in (b) and (c) were considered BrdU-labeled cells. Scale bar = 20 μm .

Analysis

A researcher blind to treatment and sex counted GAL-ir cells with and without BrdU immunoreactivity under bright-field illumination. GAL-ir cells were counted at levels corresponding to plates 19, 20, 21, and 22 (BST) and plates 27, 28, 29, and 30 (CMA) of the atlas of Paxinos and Watson (1988). Every GAL-ir or AVP-ir cell body with a visible nucleus, either clear or labeled with BrdU, was counted. Also noted was the degree of BrdU labeling, which ranged from none [Fig.1(a)] to light (spotty cytoplasmic to light nuclear staining) [Fig.1(b)], to dark (dense nuclear staining) [Fig.1(c)]. Images were captured from the microscope using 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. To count the number of BrdU-labeled nuclei, sections were analyzed using NIH Image 1.47 (W. Rasbaud, NIH). Background and foreground labeling were defined using gray-level thresholding such that most GAL-ir cell bodies were not detected as foreground (Shipley et al., 1989). Additional GAL-ir cells were excluded by defining BrdU-labeled cells by size. Only thresholded particles with a size between 10 and 120 pixels (8.1–97.6 μm^2) were counted as positive cells in a 410×240 -pixel (333×195 - μm^2) box overlying a region of GAL-ir cell bodies in the BST; the same was done in a 340×340 -pixel (276×276 - μm^2) box in the CMA. To determine GAL-ir cell size, the transsectional area of three randomly chosen cell profiles in the BST and in the CMA in randomly chosen males and females ($n = 6/\text{group}$) was measured using NIH Image 1.47.

The total number of GAL-ir cells was compared between males and females using Student t test and differences in the percentage of cells immunostained for both GAL and BrdU to total GAL-ir cells were tested using two-way analysis of variance (ANOVA) with sex, day of labeling, and/or area as factors. A significance level of $p = .05$ was used, followed by a Student–Newman–Keuls post hoc test to determine the nature of the differences. Differences in the number of cells labeled with BrdU over different dates and between sexes were also determined using two-way ANOVA.

RESULTS

Because of possible teratogenic effects of multiple injections of BrdU (Skalko, 1975; Franz and Kleinebrecht, 1982), we limited BrdU administration to one injection at a dose that might not label all cells that were born at each date examined. However, the observed pattern of BrdU labeling reflected reported patterns of neurogenesis. We found that the greatest number of cells in the BST and CMA were labeled with BrdU on E14 and E15 (Fig. 5), consistent with previous work using tritiated thymidine (Bayer, 1980, 1987). Also, we observed double labeling in areas such as the SON and PVN during periods when neurogenesis have been reported to occur in those areas (data not shown) (Bayer and Altman, 1995).

As an additional control for the consistency of the present experiment with previous studies, one series of sections was immunostained for AVP and BrdU. Two to three animals from each sex and date were analyzed such that 38 animals were included (2 males and 2 females for E11 and E12 and 3 males and 3 females for E13–17 for each time point). Consistent with previous findings (Al-Shamma and De Vries, 1996), more than 80% of all BrdU-labeled cells were found in animals exposed to BrdU on E12 and E13 for both males and females. No AVP-ir cells were labeled on E11.

Galanin Immunoreactivity in the BST and CMA

The number of GAL-ir cells in the BST and CMA was comparable to those stained with a nickel-intensified method used in other studies in our laboratory, suggesting that there was no significant

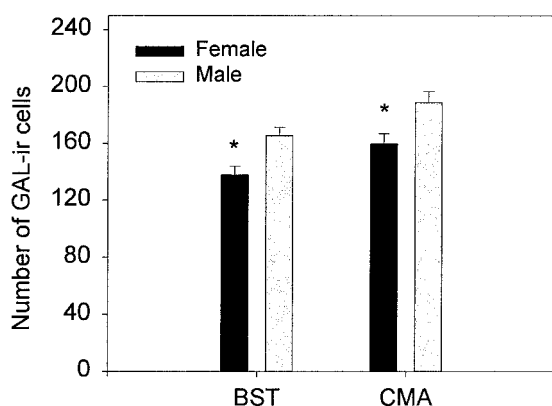


Figure 2 Number of GAL-ir cells counted in every fourth section throughout the BST and CMA of male and female rats treated prenatally with BrdU (50 mg/kg). Bars indicate mean values \pm S.E.M. Asterisks indicate that males exhibited more GAL-ir cells than did females in the BST (*t* test, $p < .05$) as well as in the CMA (*t* test, $p < .005$).

loss in detectability of GAL-ir cells in the double-labeling procedure. The number of GAL-ir cells in the BST/CMA with and without BrdU labeling were counted using every fourth section in a total of 86 animals (44 females, 42 males). Student *t* test demonstrated a slight but significant sex difference in GAL expression (Fig. 2) in both the BST [mean \pm standard error of the mean (S.E.M.); male, 157.6 ± 5.1 cells; female, 142.2 ± 5.5 cells, $p < .05$] and CMA (male, 186.0 ± 5.0 cells; female, 166.0 ± 4.0 cells, $p < .005$). Also, the CMA contained more GAL-ir cells than did the BST (male, $p < .0005$; female, $p < .005$). A two-way ANOVA revealed no differences in GAL-ir cell profile size based upon sex and area. The average cell profile was $342.5 \pm 26.3 \mu\text{m}^2$ and $328.3 \pm 54.3 \mu\text{m}^2$ in the BST of females and males, respectively, and $346.0 \pm 21.8 \mu\text{m}^2$ and $335.3 \pm 26.7 \mu\text{m}^2$ in the CMA of females and males, respectively.

Galanin-ir cell bodies were distributed in a manner typical of AVP-ir cell distribution in the BST/CMA (De Vries et al., 1985). The number of GAL-ir cell bodies in the BST increased rostro-caudally. In the rostral BST, GAL-ir cells were sparsely distributed within the anterior medial division. At more caudal levels, the number of GAL-ir cells peaked within a crescent-shaped region in the posterior lateral and medial posteromedial division of the BST. In the CMA, GAL-ir cells were concentrated in the anterior portion in the intra-amygdaloid division of the BST and the laterodorsal aspect of the medial amygdaloid nucleus.

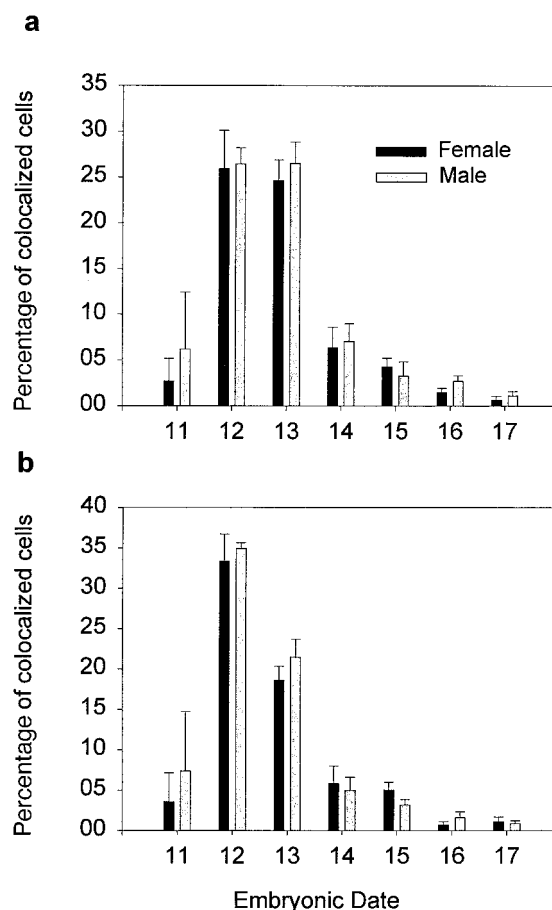


Figure 3 GAL-ir cell birth profile in the BST (a) and CMA (b). Bars indicate mean \pm S.E.M. There was a significant interaction between date and area (ANOVA, $p < .0001$). The post hoc analysis indicated that more GAL-ir cells were generated on E12 and E13 in the BST and CMA than on any other date. In addition, in the CMA, more GAL-ir cells were generated on E12 than on E13.

Colocalization of BrdU and Galanin in the BST/CMA

A total of 79 animals were included in the analysis (E11: 5 females and 3 males; E12: 6 females and 5 males; E13–17: 6 females and 6 males at each time point). We found no significant effects of area or sex on the percentage of GAL-ir cells in animals exposed to BrdU between E11 and E17. We found a significant effect of day of injection, however [ANOVA, $F(6, 63) = 70.0$, $p < .0001$]. In both the BST and CMA, GAL-ir cells could be labeled from E11 to E17, but the greatest percentage of GAL-ir cells was labeled on E12 and E13 (Fig. 3). In the BST, the mean percentage (\pm S.E.M.) of GAL-ir cells labeled with BrdU was 26.2 ± 1.4 for animals exposed to BrdU on E12 and 25.6 ± 1.3 for those exposed on E13. For the CMA,

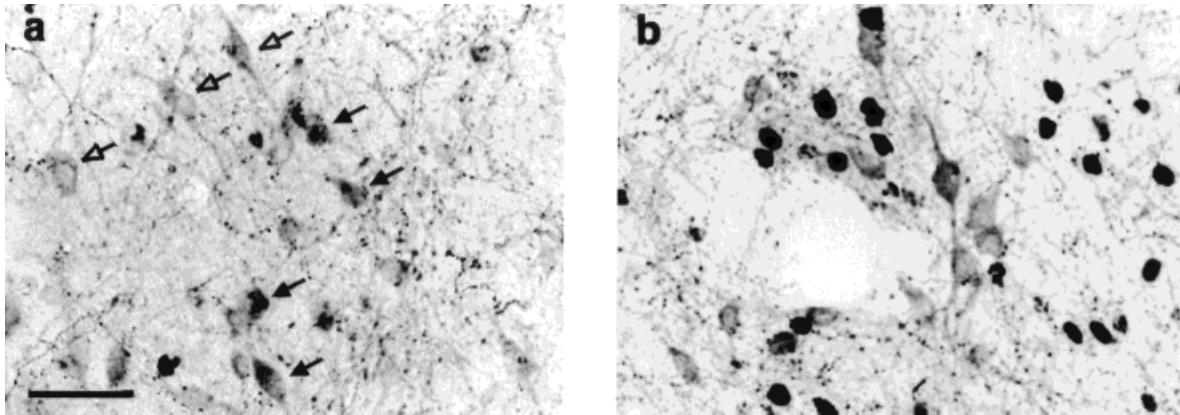


Figure 4 Photomicrographs of BrdU labeling in the BST in adult animals that were exposed to BrdU on embryonic days 12 (a) and 14 (b). Solid arrows indicate BrdU-labeled GAL-ir cells; open arrows indicate unlabeled GAL-ir cells. Scale bar = 50 μ m.

these percentages were 34.2 ± 1.1 on E12 and 20.2 ± 1.1 on E13. Labeling on E11 was found in only one male and female in the litter of only one dam. These subjects exhibited considerable colocalization of GAL and BrdU at about 50% of the level observed in animals exposed to BrdU on E12.

A two-way ANOVA demonstrated a significant interaction between the day of BrdU injection and area [ANOVA, $F(6, 140) = 5.7, p < .0001$]. A Student–Newman–Keuls post hoc test showed no significant difference between the percentage of GAL-ir cells labeled on E12 and E13 in the BST [Fig. 3(a)], but a greater percentage of cells on E12 than on E13 in the CMA [Fig. 3(b)].

We found a significant effect of day of injection on the total number of cells labeled with BrdU in the area that contains the GAL-ir cells in the BST [ANOVA, $F(6, 70) = 51.2, p < .0001$] and CMA [ANOVA, $F(6, 70) = 52.9, p < .0001$]. The overall pattern of BrdU labeling in the cells of the BST/CMA was different from the patterning of BrdU labeling in GAL-ir cells. While the percentage of BrdU-labeled GAL-ir cells peaked in animals exposed to BrdU on E12 and E13, the number of all BrdU-labeled cells in the area that contained the GAL-ir cells peaked in animals exposed to BrdU on E14 and E15 (Figs. 4 and 5). There was no interaction between sex and date and no interaction between area and date in the total number of BrdU-labeled cells. However, the number of BrdU-labeled cells in the sampling area in the CMA was slightly but significantly higher in females than in males (Fig. 6) [ANOVA, $F(6, 76) = 9.5, p < .005$].

Galanin-ir cells in areas other than the BST/CMA showed a different pattern of labeling. For example, GAL-ir cells in the supraoptic nucleus and the para-

ventricular nucleus were labeled with BrdU mainly on E14.

DISCUSSION

This study characterized the cell birth profile of GAL-ir cells in the BST/CMA. In both the BST and CMA, the greatest percentage of GAL-ir cells was generated between E12 and E13, whereas the greatest percentage of all cells in the same area that contained these GAL-ir cells was generated between E14 and E15. This cell birth profile is similar to what has been

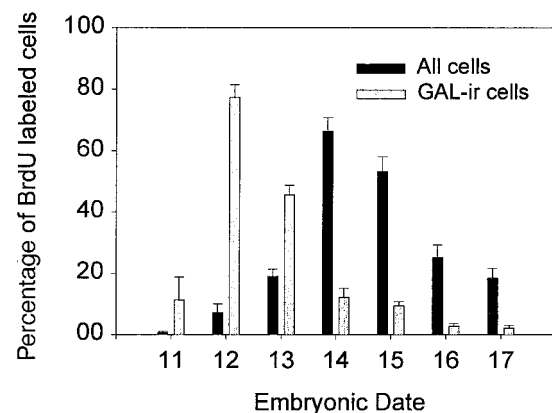


Figure 5 Comparison of normalized cell birth profiles for all cells versus GAL-ir cells in the CMA. Data were normalized using the largest value of BrdU-labeled or colocalized cells for all cells and for all GAL-ir cells, respectively. Male and female values were pooled. Cell birth of all cells in the CMA peaked on E14 and on E15; cell birth of GAL-ir cells peaked at E12 and E13. Analysis of the BST shows a similar profile.

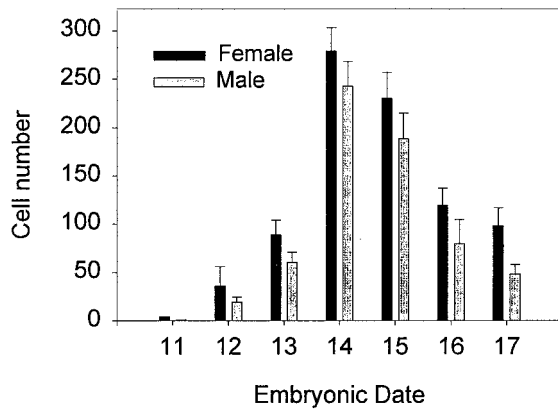


Figure 6 Comparison of the number of BrdU-labeled cells in the CMA of males and females. There was a significant effect of sex (ANOVA, $p < .05$); females showed consistently greater numbers of BrdU-labeled cells in the CMA.

described previously for AVP-ir cells (Al-Shamma and De Vries, 1996). Furthermore, the degree of BrdU-labeling of GAL-ir cells was the same between males and females, further supporting the idea that the population of GAL-ir cells is equivalent between sexes. Hence, the results presented here are consistent with the hypothesis that sexual differentiation of AVP-expressing cells in the BST/CMA involves the differential induction of AVP expression by GAL cells.

The similarity in cell birth profile of GAL-ir cells observed in the present study and of AVP-ir cells observed previously (Al-Shamma and De Vries, 1996) does not allow us to rule out the possibility that sex differences in developmental processes other than differentiation of neuronal phenotype contribute to sex differences in AVP expression. For example, it remains possible that there are two distinct populations of GAL-expressing cells (GAL+AVP and GAL-only), which are born simultaneously. If, in that case, sex differences in programmed cell death contributed to sex differences in AVP expression, males and females must have inverse patterns of cell death: The number of GAL-only cells in males that die would equal the number of GAL+AVP cells that die in females. This would be consistent with the absence of sex differences in the cell birth profile of GAL-ir cells observed in the present study. To test whether differences in neuronal cell death play a role in the development of sex differences in this system, additional studies are needed to test for sex differences in programmed cell death of cells that will express GAL+AVP. Currently, there are no markers that can distinguish between GAL-expressing cells that will coexpress AVP and those that will not. However, the

most parsimonious explanation for the present findings is that the sex difference in GAL and AVP coexpression is based on the differential induction of an AVP-ir phenotype in a subset of GAL-ir cells. Such a sex difference in the development of neurotransmitter phenotype may be a common feature of sexually dimorphic structures. Male ferrets, for example, have more GAL-immunoreactive cells in the dorsal preoptic area/anterior hypothalamus than do females (Park et al., 1997). As there is no evidence for sex differences in neurogenesis, cell migration, or cell death in this area (Park et al., 1998), the sex difference in GAL expression may be based on sexual differentiation of neuronal phenotype as well.

Although previous studies have found no sex difference in GAL mRNA expression, we demonstrate here that males possess more GAL-ir cells than do females (Fig. 2). The sex difference in GAL peptide expression is slight, with females exhibiting approximately 10% fewer GAL-ir cells than males. This sex difference in peptide expression may have become apparent owing to the large number of subjects analyzed. Because the level of GAL mRNA expression per cell is regulated by gonadal steroid hormones (Miller et al., 1993a; Planas et al., 1994), sex differences in circulating hormones may have caused a sex difference in the level of GAL peptide expression and consequently, in the level of GAL immunoreactivity. Although Planas et al. (1995) did not find a difference in the total number of GAL mRNA-expressing cells, even though they used intact animals as well, it is still possible that unknown effects of gonadal hormones on posttranslational processing of preprogalanin may have contributed to the sex difference in GAL-ir cell number. It is important, however, to note that this sex difference is too small to explain the sex difference in AVP coexpression. In males about 60% of all GAL mRNA-expressing cells coexpress AVP mRNA; in females, this percentage is about 40% (Planas et al., 1995).

We also found that the BST and CMA differed in the cell birth profiles of GAL-ir cells. While the percentage of GAL-ir cells labeled with BrdU in the BST did not differ between animals exposed to BrdU on E12 and E13, this percentage was significantly greater for those exposed on E12 than on E13 in the CMA (Fig. 2), suggesting that the CMA is composed of a set of older GAL-ir cells than is the BST. The greater percentage of older GAL-ir cells in the CMA versus the BST supports the idea that GAL-ir cells in the BST/CMA arise from the neuroepithelial zone at the base of the lateral ventricles and migrate ventrolaterally such that older cells will settle in more distant sites from the lateral ventricles than younger cells

(Bayer, 1980, 1987; Bayer and Altman, 1995). Moreover, the relatively younger age of GAL-ir cells in the BST compared with the CMA may explain why treating neonatally castrated males with testosterone propionate on postnatal day 7 (P7) increased the number of AVP-ir cells only in the BST and not in the CMA (Wang et al., 1993). At P7, the older cells in the CMA may have passed the period during which they can still respond to testosterone by altering their phenotype.

We found unexpectedly that the total number of BrdU-labeled cells in the CMA of females was consistently greater than that in males (Fig. 6). Because the cell counts were done in similarly sized areas in males and females, and because this difference was consistent across all dates examined, this sex difference in BrdU-labeling may be based on a sex difference in cell density. Overall cell density may be greater in the region of the CMA that contains GAL-ir cells in females than in males. Although sex differences in volume of the principal nucleus of the BST and the medial nucleus of the CMA have been described (Del Abril et al., 1987; Hines et al., 1992; Mizukami et al., 1983), sex differences in cell density in these areas have not been reported and warrant further investigation.

An anomaly in the present study was that subjects from one litter injected with BrdU at E11 showed significant labeling at levels above those observed in other E11 subjects in this and the previous study. The time of insemination for this litter may have occurred a few hours earlier than for the other litters used for this time point such that the BrdU labeled the neurogenesis of an early wave of galaninergic cells.

The mechanisms by which GAL-ir cells acquire AVP expression is unknown. Differentiation of neurotransmitter phenotype has been most thoroughly studied in the peripheral nervous system. For example, sympathetic neurons innervating the sweat glands in the rat are initially noradrenergic, but upon contacting their targets become cholinergic (Habecker and Landis, 1994), presumably due to the secretion of a cholinergic differentiation factor by the sweat glands (Habecker et al., 1995). Whether similar target-dependent mechanisms are operating in sexual differentiation of neurotransmitter phenotype in the central nervous system or whether other mechanisms are more important remains to be determined. However, target-dependent mechanisms may play a role in the differentiation of the BST/CMA. From P10 onward, there is a very sparse projection from the principal nucleus of the BST to the anteroventral periventricular nucleus of the hypothalamus in females, whereas this projection is about 20-fold more dense in

males (Hutton et al., 1998). This difference could expose BST cells, including the GAL cells, to difference target-derived factors in males and females. Studies on the development of the sexually dimorphic AVP expression by GAL cells offer the opportunity to elucidate cellular mechanisms underlying differentiation of neuronal phenotype.

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