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Increased Number of BrdU-Labeled Neurons in the Rostral Migratory Stream of the Estrous Prairie Vole

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In the mammalian forebrain, most neurons originate from proliferating cells in the ventricular zone lining the lateral ventricles, including a discrete area of the subventricular zone in which neurogenesis continues into adulthood. The majority of the cells generated in the anterior portion of the subventricular zone (SVZa) are neuronal precursors with progeny that migrate to the olfactory bulb (OB) along a pathway known as the rostral migratory stream (RMS). The list of factors that influence the proliferation and survival of neurons in the adult brain remains incomplete, but previous studies have implicated neurotrophins in mammals and estrogen in birds. This study examined the effect of estrus induction on the proliferation of SVZa neurons in female prairie voles. Prairie voles, unlike many other rodents, are induced into estrus by chemosensory cues from a male. This olfactory-mediated process results in an increase in serum estrogen levels and the consequent induction of behavioral estrus (sexual receptivity). Female prairie voles induced into estrus by male exposure had a 92% increase in BrdU-labeled cells in the SVZa compared to females exposed to a female. Double-label immunocytochemical studies demonstrated that 80% of the BrdU-labeled cells in the RMS displayed a neuronal phenotype. Ovariectomized females exposed to a male did not show an increase in serum estrogen or BrdU labeling in the RMS. Conversely, ovariectomized females injected with estrogen were sexually receptive and had more BrdU-labeled cells in the RMS than oil-injected females. These data suggest that, in female prairie voles, estrus induction is associated with increased numbers of dividing cells in the RMS, possibly via an estrogen-mediated process.

Key Words: **estrogen; BrdU; subventricular zone; neurogenesis; subependymal zone.**

The subventricular zone, lining the lateral ventricle, is one of a number of brain regions where neuronal birth and migration continue into adulthood (Altman, 1969; Kaplan and Hinds, 1977). Previous studies have demonstrated that cells born in the anterior portion of the subventricular zone (SVZa) include neuroblasts that migrate to the olfactory bulb (Luskin, 1993). These neuroblasts are organized as chords of cells (Lois *et al.*, 1996) that migrate tangentially along a highly defined route called the rostral migratory stream (RMS) (Luskin, 1993; Lois and Alvarez-Buylla, 1993a). SVZa-derived neuroblasts express a neuronal phenotype while maintaining the capacity to divide throughout the course of their migration to the olfactory bulb (Menezes *et al.*, 1995; Lois and Alvarez-Buylla, 1993b). Once in the olfactory bulb, these cells migrate radially and differentiate into GABAergic and dopaminergic interneurons of the glomerular and granule cell layers (Betarbet *et al.*, 1996).

Although the evidence for continued neurogenesis in the SVZa and RMS is now strong, the range of factors that influence this process and its functional importance remain largely unknown. Several lines of evidence suggest that estrogen may influence cell number by altering the proliferation rate and/or the survival of newly generated neurons in other brain regions. For example, previous work has demonstrated that estrogen plays an important role during the organization of sexually dimorphic brain areas (Arnold and Gorski, 1984; Toran-Allerand, 1976, 1980, 1983). In adult rats, estrogen increases dendritic spine formation, synaptogenesis, and neurogenesis in the hippocampal formation (Gould *et al.*, 1990; Woolley and McEwen, 1992; Tanapat *et al.*, 1999). The rate of

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cell proliferation in the dentate gyrus peaks during proestrus, when estrogen levels are highest, and ovariectomy diminishes the rate of neurogenesis in this region (Tanapat *et al.,* 1999). In song birds, estrogen and testosterone promote the survival of neurons that originate in the ventricular zone (Burek *et al.,* 1994, 1995; Hildago *et al.,* 1995). Finally, estrogen regulates the expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and possibly other neurotrophins which may promote the proliferation and/or survival of SVZa-derived cells in adult rats (Kirschenbaum and Goldman, 1995; Singh *et al.,* 1995; Zigova *et al.,* 1997; Sohrabji *et al.,* 1994; Kuhn *et al.,* 1997). These data suggest that estrogen may act via growth factors to regulate neurogenesis (Sohrabji *et al.,* 1994, 1995) and that endocrine status may impact the proliferation of SVZa-derived progenitor cells in the adult brain.

In this study, we sought to determine whether the proliferation of SVZa-derived neurons is influenced by estrogen and specifically by the induction of estrus in the female prairie vole. Unlike rats, female prairie voles lack an estrous cycle and are induced into estrus by chemosensory cues from unfamiliar adult male voles (Richmond and Conaway, 1969). Virgin female prairie voles remain sexually immature unless exposed to a male for approximately 24 to 48 h, resulting in the induction of behavioral estrus, characterized by sexual receptivity to a male. Estrus induction is associated with profound hormonal changes, including a rise in serum estrogen levels as well as increased estrogen receptor binding in the brain (Cohen-Parson and Carter, 1987; Dluzen *et al.,* 1981; Carter *et al.,* 1987a; Hnatczuk and Morrell, 1995). Thus, the prairie vole provides an excellent opportunity to study whether the proliferation of SVZa-derived neuroblasts increases with the onset of sexual maturity and/or with changes in serum estrogen levels. We hypothesized that estrus induction would increase the proliferation of SVZa-derived cells and that estrogen, in part, may mediate this process.

MATERIALS AND METHODS

Animal Care and Treatment

Adult prairie voles (*Microtus ochrogaster*) were the F2 generation of a breeding colony started with field-captured animals ranging in age from 60 to 75 days. Subjects were weaned at 21 days of age and housed with same-sexed siblings in plastic cages (20 × 25 × 45 cm) that contained cedar chips. Test animals were sexually naïve females. Stimulus animals were either sexually experienced adult male prairie voles or sexually naive adult female prairie voles from our colony maintained under identical housing conditions. All cages were maintained on a 14:10 h. light:dark photoperiod at approximately 21°C. Food and water were provided *ad libitum.*

Procedure

Experiment I: Intact females exposed to males. At 60–70 days of age, female subjects were housed with siblings, meaning one or two sisters from their litter ($n = 6$), an adult female from an unrelated litter ($n = 5$), or a sexually experienced, unrelated male ($n = 11$) for 48 h. The unrelated female group was included to control for exposure to a novel conspecific. The 48-h cohabitation was used in light of pilot data and previous reports suggesting that a 48-h cohabitation was sufficient to induce estrus (Carter *et al.,* 1987a). In each pair, the subject was separated from the stimulus animal by a fine wire mesh which allowed the animals to see, smell, and have limited physical contact with each other, but prevented them from mating. All females were injected with the cell proliferation marker 5-bromo-2′-deoxyuridine (BrdU) every 6 h for the final 24 h of the 48 h of cohabitation. This schedule was found to give the highest number of BrdU-labeled cells in pilot studies comparing single point injections with repeated injections as well as injections in the first 24 h vs. the second 24 h of male exposure. Injections of BrdU (Sigma) were given intraperitoneally (50 μg/g body weight) in 0.9% saline and 0.007 N NaOH (the total amount of BrdU administered was 6.25 mg over 24 h). At the end of the 48-h cohabitation period, females in all groups were tested for sexual receptivity to determine if they were in estrus. Testing consisted of placing a male in the female's cage. The pair remained together until the female showed lordosis, or for 10 min, whichever came first. If the female was not receptive to the male, the male was removed and a second male was introduced for an additional 10 min. Sexual receptivity, assessed by the presence of lordosis, was evident in 63.5% (7 of 11) of subjects housed with a male for 48 h. Of the female subjects housed with a male, only the 7 showing lordosis were considered in estrus and only these females, with females from the other two groups, were sacrificed, perfused, and processed for immunocytochemistry. No experimental female housed with an adult female stimulus animal or with a sibling was sexually receptive. An

additional group of two male-exposed estrous and two sibling-exposed nonestrous females were used for analysis of phenotype (see below).

Experiment II: Ovariectomized females exposed to males. Male exposure leading to estrus induction is associated with a threefold increase in serum estrogen levels in prairie voles (Cohen-Parson and Carter, 1987). To determine if the BrdU incorporation observed following male exposure occurs in the absence of estrogen, the above experiment was repeated with ovariectomized female prairie voles. Two weeks after ovariectomy, females were housed with female siblings ($n = 5$), unrelated females ($n = 5$), or a sexually experienced male ($n = 6$). Subjects and stimulus animals were separated by a fine wire mesh. Animals were housed together for a total of 48 h and a single BrdU injection (50 μg/g body weight) was given every 6 h during the final 24 h of the 48-h cohabitation (the total amount of BrdU administered was 6.25 mg over 24 h). Six h after the final BrdU injection, experimental females were sacrificed, perfused, and processed for immunocytochemistry.

Experiment III: Ovariectomized females treated with estradiol benzoate. In order to determine if estrogen stimulates increased cell proliferation of SVZa-derived cells, we examined the effects of estrogen administration in a separate group of female voles. Sexually naive female subjects were ovariectomized, allowed 14 days to recover, and then treated with either EB (β-estradiol 3-benzoate; 1 μg, $n = 9$) or the vehicle alone (sesame oil, $n = 5$). Three injections, of either estrogen or oil, were given at 24-h intervals. All animals received a single injection of BrdU (50 μg/g body weight) every 6 h for 24 h following the final injection of estrogen or oil (the total amount of BrdU administered was 6.25 mg over 24 h). After the 24 h of BrdU injections, all estrogen-treated animals were tested for sexual receptivity, as described above. Of the females given EB injections, only females that were sexually receptive (5 of 9) were sacrificed, perfused, and further processed for immunohistochemistry.

Serum Estrogen Assays

Immediately after behavioral testing for lordosis, blood samples were obtained by using a syringe to draw blood from the jugular vein. Blood was collected on ice in centrifuge tubes and centrifuged at 4000 rpm for 10 min. The resulting serum was stored at −80°C. Serum concentrations of estradiol were measured by double antibody radioimmunoassay using commercially available reagents (Diagnostic Products Corp., Los Angeles, CA). Following extraction of serum with diethyl ether to remove serum matrix effects, the organic phase was dried under a stream of N_2 and reconstituted with Kit's diluent. Using an equivalent of 100 μl of serum in duplicate, the assay had a sensitivity of 5.0 pg/ml, corresponding to 92% binding compared to reference (B/B_0) and having displacement that was 6.2 SD from that observed for reference tubes. The upper limit of the assay was 1000 pg/ml at 14% B/B_0. Assaying increasing volumes of the serum extract from 2.5 to 100 μl produced a displacement line parallel to the standard curve. Intra-assay and interassay coefficients of variation (CVs) averaged $<$5.0 and 6.3%, respectively.

Histological and Immunohistochemical Procedures

At the end of the cohabitation, female subjects were anesthetized with sodium pentobarbital (0.03 ml/10 g body weight) and perfused through the ascending aorta with 20 ml of 0.9% saline followed by 100–150 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were removed, stored in 4% paraformaldehyde for 24 h, transferred to 20% sucrose for an additional 24 h, and sectioned in the sagittal plane at 35 μm with a microtome.

Detection of BrdU

Floating sections at 140-μm intervals were processed for BrdU immunostaining. Sections were treated with 2 N HCl for 30 min in a 60°C water bath to denature the DNA and then with borate buffer (pH 8.3) for 25 min. After rinsing in 0.1 M PBS, sections were incubated in 0.3% hydrogen peroxide in methanol for 15 min and then treated with 10% goat serum and 0.5% Triton X-100 in 0.1 M PBS (blocking serum) for 1 h. Sections were incubated in rat anti-BrdU monoclonal antibody (Accurate, Westbury, NY) 1:500 in blocking serum overnight at 4°C and then rinsed and incubated in biotinylated goat anti-rat IgG 1:200 in blocking serum for 1 h at room temperature. Finally, sections were incubated in ABC Vector Elite in 0.1 M PBS for 1 h and immunoreactivity was revealed using the chromagen diaminobenzidine (DAB). To reduce variability in the background and standardize the staining, sections from all subjects in each experiment were processed concurrently for BrdU immunohistochemistry. Controls for BrdU immunocytochemistry consisted of processing brain sections from voles

that did not receive BrdU injections and processing brain sections from animals that received BrdU injections but were not treated with the anti-BrdU antibody.

Determination of Cell Phenotype in BrdU-Labeled Cells

Brains were embedded with O.C.T. (Miles Inc., Elkhart, IN) and frozen using liquid nitrogen. Twenty-micron-thick sagittal sections were thaw mounted onto Superfrost slides (Fisher Scientific, PA). Slides were incubated at 4°C with the monoclonal antibody to BrdU (Accurate) at a dilution of 1:200 in combination with a cell type-specific antibody. To identify neurons, we used a rabbit polyclonal antibody recognizing β-tubulin type III (TuJ1, Covance, Richmond, CA) at a dilution of 1:200 (Lee *et al.*, 1990a,b). To identify astrocytes, we used an antibody to glial fibrillary acidic protein (GFAP, Dako) at a dilution of 1:500. After an overnight incubation in the primary antibodies, the sections were rinsed in buffer and incubated for 1 h in a secondary antibody cocktail formed by lissamine–rhodamine-conjugated goat anti-rat antibody for BrdU and FITC-immunoreactive goat anti-rabbit antibody to visualize the TuJ1 or GFAP; all secondary antibodies were from Jackson ImmunoResearch (PA). Following the second antibody reaction, sections were coverslipped with Vectashield (Vector, CA). Control sections were generated in all cases by omitting the primary antibodies. For a cell to be counted as double labeled for BrdU and either TuJ1 or GFAP, we required that the neuronal or glial label was detectable throughout the cytoplasm. Using these criteria, the nuclei of neurons or astrocytes were easily distinguished even without BrdU incorporation. As astrocytes were usually non-overlapping in the RMS, the risk that two cell bodies might mask each others' nuclei was very small. However, to eliminate this possibility, we used confocal microscopy.

Data Analysis

All cell counts were performed blind to treatment. In each of the three experiments, we counted the number of BrdU-positive cells in three subdivisions of the RMS: the SVZa (corresponding to the expanded region adjacent to the lateral ventricle), the intermediate portion (IP) of the RMS (rostral to the SVZa and caudal to the entrance to the olfactory bulb, encompassing the descending and longitudinal limbs of pathway), and the subependymal zone in the core of the olfactory bulb (sezOB) (Menezes *et al.*, 1995) (Fig. 1). Labeled cells were counted with a 40X objective in each of the three brain regions using the NIH Image 1.54 image analysis software. Six to eight sections distributed evenly across the mediolateral axis of the rostral migratory stream were counted per animal, with the most medial section corresponding to the Paxinos and Watson *Rat Brain Atlas* (1982), Fig. 45, through the descending limb of the fornix and the most lateral section corresponding to the Paxinos and Watson *Rat Brain Atlas*, Fig. 47, through the lateral septum. Only sections in which the entire rostral migratory stream was visible were analyzed. Relative cells/field were averaged over the number of sections analyzed to obtain the mean number of BrdU-labeled cells for each region per subject. Treatment effects for each brain region were analyzed using a one-way ANOVA. Significant differences were further evaluated using a Student–Newman–Keul's test. In the estrogen treatment experiment, which had only two groups, treatment effects were examined using a two-tailed, unpaired *t* test. To calculate the percentage of newly generated cells that express a neuronal or glial phenotype, two male-exposed and two sibling-exposed females from Experiment I were used. For these two groups, we counted at least 300 cells in three to six sagittal sections (20–40 μm apart) along the rostral–caudal extent of the RMS using both light and confocal microscopy.

RESULTS

Experiment 1

Of the three groups (exposed to male, novel female, or sibling), only females exposed to males entered behavioral estrus (see Materials and Methods). In all of the groups, BrdU-labeled cells were present throughout the RMS, allowing us to examine the migration and distribution of mitotic SVZ-derived cells in the prairie vole. BrdU immunostaining was exclusively nuclear within the three sudivisions of the RMS (SVZa, IP, and sezOB), with few labeled cells observed outside the pathway (Fig. 1).

Estrous females had more BrdU-labeled cells in the SVZa, IP, and sezOB than subjects housed with a female or housed with siblings ($F = 10.174$; $P < 0.01$) (Figs. 2 and 3). Compared to sibling-housed controls, the number of BrdU-labeled cells in estrous females was increased 92% in the SVZa, 78% in the IP,

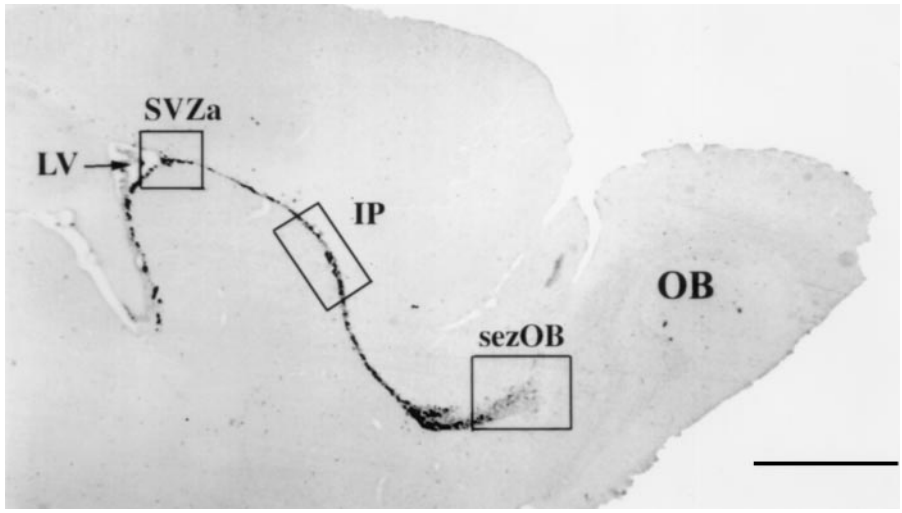

FIG. 1. Newly generated BrdU-labeled cells in the rostral migratory stream of the female prairie vole. (A) Sagittal section of a female prairie vole forebrain demonstrating BrdU-labeled cells along the rostral–caudal extent of the rostral migratory stream (RMS). BrdU was administered during the second 24 h of 48-h cohabitation with a male (see Materials and Methods). Black dots represent BrdU-labeled cells migrating to olfactory bulb. Scale bar, 1 mm. Blocks demarcate the anterior subventricular zone (SVZa) surrounding the anterior tip of the lateral ventricle (LV) and the distal portion of the RMS, which we denote as the subependymal zone (sez) of the olfactory bulb (OB).

and 103% in the sezOB. Females housed with a novel female did not differ from those housed with their siblings in the number of BrdU-labeled cells in any of the measured brain regions. Thus, the increase in BrdU-labeled cells thoughout the RMS was specific to female subjects induced into estrus by exposure to a

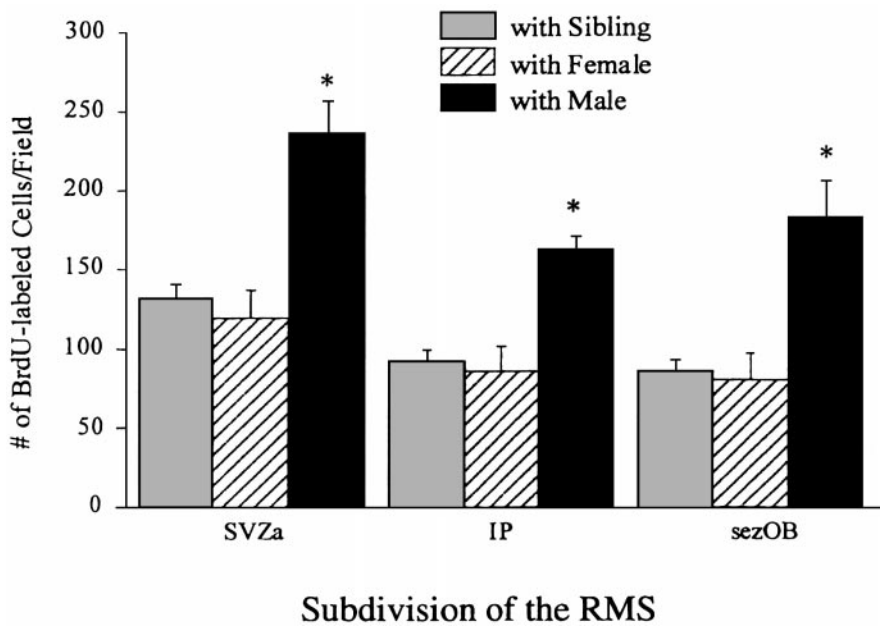

FIG. 2. A comparison of the number of BrdU-labeled cells in female prairie voles housed with a male, female, or female sibling. BrdU was administered during the second 24 h of the 48-h cohabitation. Male-exposed females which became sexually receptive had significantly more BrdU-labeled cells in each of the three regions analyzed than either female-exposed group. Subjects housed with their same-sex siblings did not differ from subjects housed with an unfamiliar female in the number of BrdU-labeled cells. Asterisks represent significant differences ($P < 0.05$). Abbreviations used: SVZa, anterior subventricular zone; IP, intermediate pathway of the RMS; sezOB, subependymal zone of the olfactory bulb.

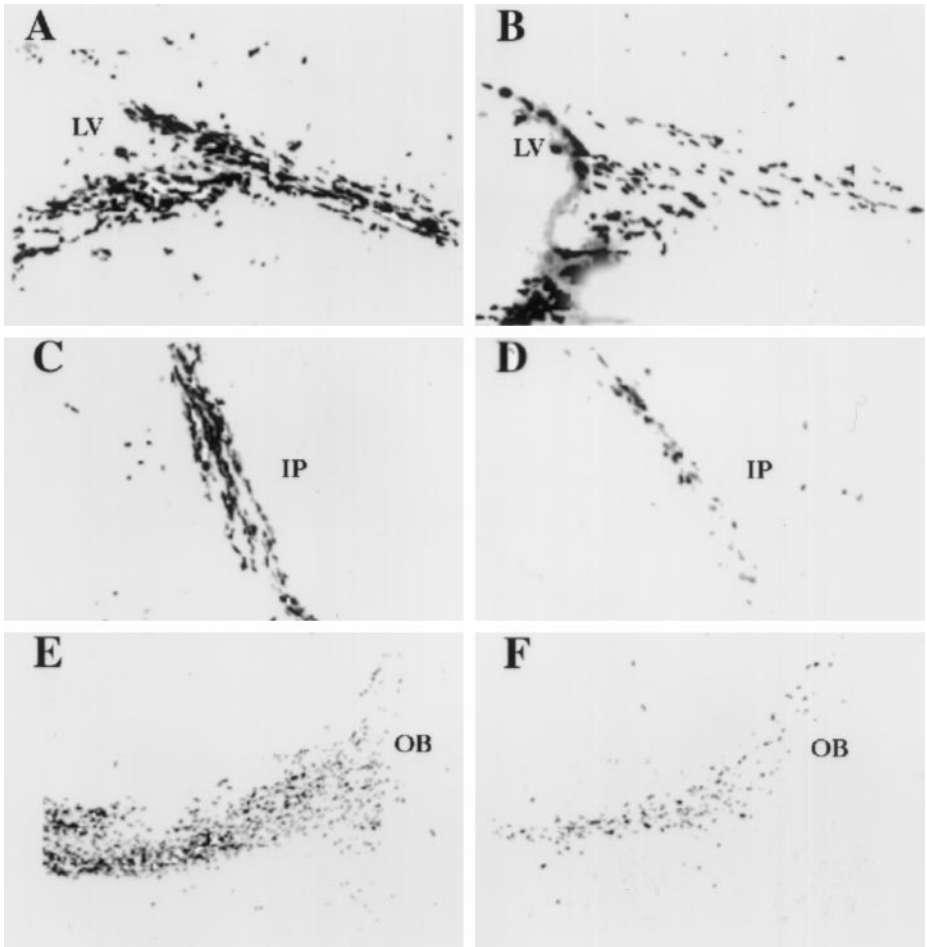

FIG. 3. Increased number of BrdU-labeled cells in the RMS of estrous females. Representative photomicrographs of the SVZa (A, B), intermediate pathway of the RMS (C, D), and subependymal zone of the olfactory bulb (E, F) demonstrate that an estrous female housed with a male (A, C, E) has significantly more BrdU-labeled cells in each region than a female housed with female siblings (B,D,F). These are the same regions shown in Fig. 1. Figures A–D were taken using a 20× objective and figures E and F were taken with a 10× objective. As shown in Fig. 2, estrous females have nearly twice the number of BrdU-labeled cells as control animals in each of the RMS regions sampled. Abbreviations used: LV, ventricle; IP, intermediate pathway of the RMS; OB, olfactory bulb.

male and was not simply the result of exposure to an unfamiliar conspecific.

To determine whether the increase in BrdU label was in neurons or glia, sections were double labeled with either an antibody to the early neuronal marker type III β-tubulin or an antibody to the glial marker GFAP. Of 368 BrdU-labeled cells counted in the estrous females, 298 (81%) were TuJ1-positive. Of 328 BrdU-labeled cells in the control females, 247 (76%) were TuJ1-positive (no statistical difference in the percentage of TuJ1-positive cells between estrous and control females) (Fig. 4). Less than 20 cells were clearly BrdU- and GFAP-positive in either estrous females or controls. Thus, the vast majority of BrdU-labeled cells were neurons, including the increased number observed in estrous females.

Experiment 2

Male exposure leading to estrus induction is associated with a threefold increase in serum estrogen levels in prairie voles (Cohen-Parson and Carter, 1987). To determine if the rise in serum estrogen associated with estrus was necessary for the observed increase in BrdU incorporation, we repeated the above experiment (sibling, unrelated female, and unrelated male exposure) with ovariectomized females. None of the ovariectomized females housed with males were

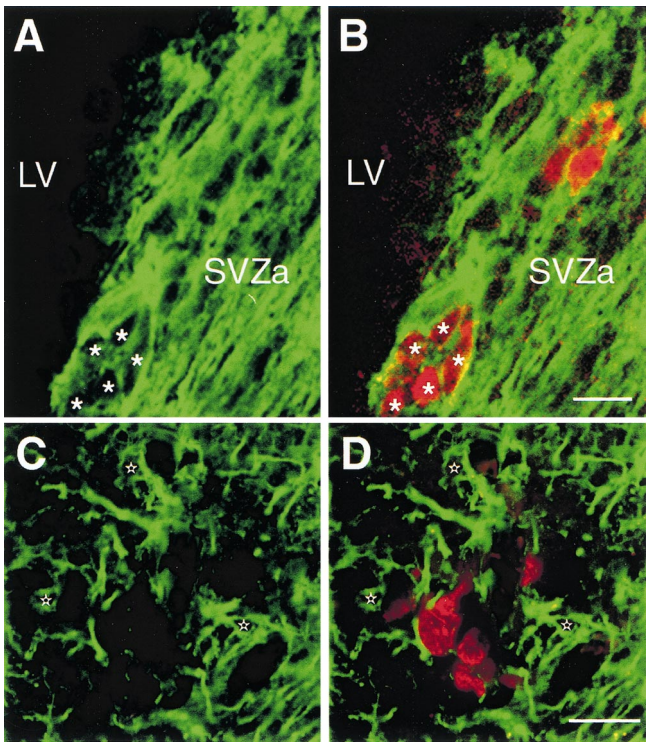

FIG. 4. Phenotype of BrdU-labeled cells in the RMS of estrous females. Sections were double labeled with an antibody to BrdU and either an antibody to the neuronal marker β-tubulin type III (TuJ1) or an antibody to the astrocytic marker glial fibrillary acidic protein (GFAP). In A, FITC—immunoreactive cells (green) represent TuJ1-positive neurons within the SVZa. In B, BrdU staining (orange) can be seen in the nuclei (asterisks) of several TuJ1-positive cells from the field shown in A. Note that the pattern of TuJ1 staining in A and B is relatively homogeneous and the cells appear to be arranged in chains. In C, FITC—immunoreactive cells (green) represent GFAP-positive astrocytes (asterisks). In D, from the same field, BrdU staining (orange) can be seen but not within the GFAP-positive cells. Note that the GFAP staining in C and D has the characteristic star burst pattern of astrocytes. The scale bar represents 20 μm, with all sections at the same magnification.

sexually receptive nor did they have elevated levels of serum estrogen levels (a mean of less than 10 pg/ml) after 48 h of cohabitation. In addition, no significant differences in the number of BrdU-labeled cells were observed between treatment groups in any of the three regions of the RMS (Fig. 5). The mean numbers of BrdU-labeled cells in the three regions of the RMS were similar to those seen in animals in the sibling housed control group in the previous experiment with intact females. These data are consistent with a role for estrogen in the observed increase in BrdU-labeled RMS cells in male-exposed intact females.

Experiment 3

To determine if estrogen alone was sufficient to increase cell proliferation in the RMS, ovariectomized females were treated with EB or oil for 3 days. Sexually receptive ovariectomized females injected with EB had an average serum estradiol level of 21.98 pg/ml (range = 17.58–30.38). In a previous report, intact females had mean serum estradiol levels of 34.9 pg/ml after 18 h of exposure to a male (Cohen-Parson and Carter, 1987). Intact females had serum estradiol levels of 13.35 pg/ml, whereas ovariectomized fe-

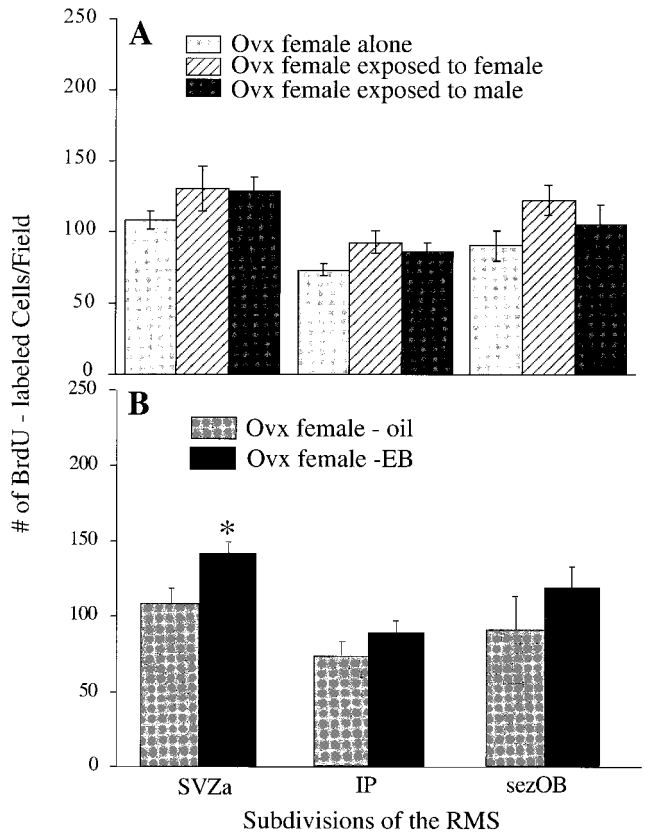

FIG. 5. A comparison of the number of BrdU-labeled cells in the RMS of ovariectomized female prairie voles. In (A), ovariectomized females housed with a male for 48 h did not show sexual receptivity nor did they have an increase in the number of BrdU-labeled cells compared to controls, suggesting that estrogen is *necessary* for the increase in BrdU labeling observed in intact female subjects housed with males (Fig. 2). In (B), ovariectomized females treated for 72 h with estradiol benzoate (EB) were sexually receptive and had an increase in the number of BrdU-labeled cells in the SVZa, but not in the IP or OB, compared to oil-treated control females. These data suggest that estrogen may, in part, be responsible for the increase in BrdU labeling observed in intact female subjects housed with males. An asterisk represents a significant difference ($P < 0.05$) between estrogen and vehicle treatments.

males injected with oil had serum estradiol levels <5.0 pg/ml. Compared to oil-treated females, ovariectomized estrous females that were given estrogen injections had a 31.5% increase in the number of BrdU-labeled cells in the SVZa ($t = 2.59$, $P < 0.05$). Interestingly, no significant differences were observed between estrogen- and oil-treated animals in the IP and the sezOB regions of the RMS, mainly due to the large variation seen between subjects.

DISCUSSION

Although previous research has described a population of mitotically active neurons in the rostral migratory stream of the adult rodent brain (Altman, 1969; Lois and Alvarez-Buylla, 1993b), little is known of the factors that influence the ongoing neurogenesis in this region. The present study demonstrates that in the female prairie vole, male-induced estrus is associated with nearly a doubling in the number of BrdU-labeled cells in the SVZa, the region of neuronal progenitor cells whose progeny migrate to the OB. This increase in BrdU-labeled cells is predominantly in cells expressing a neuronal rather than glial phenotype. We hypothesized that the elevated serum estrogen associated with estrus induction was responsible for the increased number of BrdU-labeled cells in the rostral migratory stream. Since ovariectomized females exposed to males did not show an increase in the number of BrdU-labeled cells compared to controls, our results suggest that estrogen is necessary for this phenomenon. The present study also demonstrates that exogenous estrogen treatment can increase the number of BrdU-labeled cells in the SVZa, although in contrast to male induced estrus, estrogen treatment did not increase the number of BrdU-labeled cells in more distal aspects of the RMS.

This smaller effect in the estrogen treatment study compared to that in the study of male-induced estrus is of interest. Given the modest increase (31.5%) in the SVZa and the absence of an increase in the IP or the sezOB, the most likely explanation is that the concentration of estrogen was less in the ovariectomized, estrogen-injected females. Indeed, the mean serum estrogen concentration at the time of perfusion (21.98 pg/ml) was considerably lower than that in previous reports of estrous prairie voles, suggesting that the dose of estrogen was too low. Nevertheless, we cannot rule out a role for other ovarian factors in the intact estrous females or even the possibility that male cues acting independently of the ovary might contribute to the effect observed in male-exposed, intact females. It is also possible that estrogen influences SVZa cells more than cells in the IP or sezOB zones of the RMS.

What Is the Functional Importance of an Increase in BrdU-Labeled Cells?

The functional importance of the estrus-induced proliferation of neurons is not clear. In virgin female prairie voles, male exposure induces not only estrus, but puberty. That is, females exposed to males in this experiment were induced into their first estrus and thus were, in a physiological sense, undergoing a major developmental transition. Under naturalistic conditions in this monogamous species, estrus would be followed by mating, leading to an enduring pair bond. Are the newly generated cells in the RMS important for pair bond formation? Our data do not address this question, but several observations are inconsistent with a causal relationship between neurogenesis in the RMS and pair bond formation. The pattern of newly generated cells in the RMS of the vole resemble what we and others have observed in mice and rats (Lois and Alvarez-Buylla, 1993b; Luskin 1993), species which do not form pair bonds. In the rat, these cells are destined to become interneurons of the glomerular and granule cell layers of the main olfactory bulb (Betarbet *et al.*, 1996). Based on lesion studies of the vomeronasal organ in the prairie vole, one would expect the accessory olfactory bulb rather than the main olfactory bulb to be critical for the induction of estrus (Lepri and Wysocki, 1987). Although others have described neurogenesis in the adult accessory olfactory bulb of the rat (Bonfanti *et al.*, 1997) and hamster (Huang *et al.*, 1999), we observed few BrdU-labeled cells in the accessory olfactory bulb relative to the dense staining in the main olfactory bulb of the prairie vole (unpublished data). Possibly other processes, such as dendritic outgrowth or synaptogenesis, are occurring in the absence of neural proliferation in the accessory olfactory bulb as the female becomes sexually receptive. It is also possible that the main olfactory bulb is critical for partner preference formation. Indeed, olfactory bulbectomy inhibits the development of partner preferences in female voles (Williams *et al.*, 1992), but there is little evidence that this lesion selectively blocks this behavior and there is no evidence for involvement of interneurons in the main olfactory bulb. Thus, the functional importance of the increase in BrdU-labeled cells at estrus or following estrogen treatment of female prairie voles remains unknown. Certainly, in future studies addressing the

function of this increase in cell proliferation, it will be important to count labeled cells in the main and accessory bulbs as well as their migrating precursors in the RMS.

What Is the Mechanism: Increased Neurogenesis or Decreased Cell Death?

Estrogen may increase the number of BrdU-labeled cells either by promoting the proliferation of SVZa-derived cells or by promoting the survival of newborn neurons generated in the SVZa and along the RMS. In the adult dentate gyrus, estrogen increases the rate of neurogenesis, with the number of BrdU-labeled cells 50% greater during proestrus than during estrus or diestrus when estrogen levels are lower (Tanapat *et al.*, 1999). Curiously, this increase appears transient, as these newly generated cells were no longer evident 21 days later (Tanapat *et al.*, 1999). Relative to intact females, ovariectomized females in this study had a 60% decrease in the number of BrdU-labeled cells and a twofold increase in the number of pyknotic cells in the dentate gyrus, suggesting that estrogen has important effects on survival as well as neurogenesis (Tanapat *et al.*, 1999).

In fact, estrogen effects within the RMS may be due, in part, to increased survival of cells that would normally die after their initial division. Along the route of migration a considerable number of SVZa-derived cells undergo cell death in the rat (Brunjes and Armstrong, 1996; Morshead and van der Kooy, 1992; Morshead *et al.*, 1998). Studies with conditionally immortalized cerebral cortical neuroblasts demonstrate that estrogen has complex effects on cell survival, decreasing apoptosis while transiently increasing rapid necrotic cell death in a subpopulation of cells (Wade *et al.*, 1999). Estrogen has been shown to influence the survival rather than the proliferation of cells in a number of previous studies in birds. For instance, in the zebra finch song nucleus, the HVC, estrogen promotes the survival of mitotically active neurons that would normally die, resulting in an increase in the number of dividing cells (Brown *et al.*, 1993; Burek *et al.*, 1995, 1994; Hildago *et al.*, 1995).

Since SVZa-derived cells have the capacity to divide while migrating, our results are consistent with estrogen prolonging the survival of migrating cells, allowing these cells to undergo an additional round of cell division. The cell cycle time within the RMS is roughly 14 h (Smith and Luskin, 1998), so one would expect, at most, one to two rounds of cell division in the 24 h of BrdU uptake. However, if estrogen were affecting survival rather than neurogenesis, one might expect the biggest increase in BrdU incorporation in the IP or sezOB, along the path of migration, rather than the SVZa, as observed. Furthermore, in preliminary studies, we have found only a 20% decrease in TUNEL staining (a measure of apoptosis) in the RMS of females exposed to males relative to those exposed to females (M. Smith, unpublished data). This modest decrease is unlikely to explain the doubling of BrdU-labeled cells observed in the females exposed to males. Moreover, TUNEL may label cells that are undergoing DNA repair and not undergoing apoptosis, suggesting that even this modest decrease in TUNEL staining may be overstating the change in cell death. Additional studies will be necessary to determine if the increase in BrdU incorporation reflects an increase in cell proliferation, a decrease in cell death, or a combination of both processes.

Are Estrogen's Effects Direct or Indirect?

Neurons in the prairie vole RMS lack estrogen receptors as measured by immunocytochemistry for ER-α (Hnatczuk *et al.*, 1994). Thus, it is likely that estrogen's effects on the survival of newborn cells require one or more intermediaries, such as neurotrophins (Toran-Allerand, 1996). Estrogen receptor mRNA is colocalized with mRNA for neurotrophins and their receptors in the rat forebrain (Miranda *et al.*, 1994). In addition, estrogen has been shown to increase nerve growth factor (NGF) receptor mRNA and protein levels in female rat sensory neurons and in culture (Miranda *et al.*, 1994, 1996, 1993). An estrogen response element (ERE) is found on the genes of NGF and BDNF as well as on the genes of the neurotrophin receptors trkA and p75 (Sohrabji *et al.*, 1995, 1994). BDNF may be the most intriguing of the neurotrophins containing an ERE. *In vivo*, BDNF mRNA is rapidly up-regulated in the cerebral cortex and the OB of ovariectomized animals exposed to estrogen (Sohrabji *et al.*, 1996), suggesting that estrogen can mediate the amount of BDNF present. BDNF has been shown to have dramatic effects on the proliferation and/or survival of SVZa-derived neurons. Intracerebroventricular infusion of BDNF increases the number of BrdU-labeled cells in the rat SVZa, IP of the RMS, and sezOB (Zigova *et al.*, 1996) and administration of BDNF has been shown to increase the survival of cultured rat forebrain subependymal zone neurons (Kirschenbaum and Goldman, 1995). In prairie voles, estrogen increases BDNF gene and protein expression

and thereby may indirectly increase BrdU incorporation in the RMS (Liu *et al.*, 1999).

Summary

These results, taken together, suggest that endocrine status may influence the proliferation and / or survival of neurons born in the SVZa that eventually become interneurons in the olfactory bulb. The effects are observed under physiologic conditions, when the female, stimulated by olfactory cues, exhibits sexual receptivity. Estrogen appears to be partly responsible for the increase in the number of BrdU-labeled cells in the RMS, probably by indirect effects on intermediates, such as BDNF.

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