The Effects of Social Environment on Adult Neurogenesis in the Female Prairie Vole

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ABSTRACT: In the mammalian brain, adult neurogenesis has been found to occur primarily in the subventricular zone (SVZ) and dentate gyrus of the hippocampus (DG) and to be influenced by both exogenous and endogenous factors. In the present study, we examined the effects of male exposure or social isolation on neurogenesis in adult female prairie voles (*Microtus ochrogaster*). Newly proliferated cells labeled by a cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU), were found in the SVZ and DG, as well as in other brain areas, such as the amygdala, hypothalamus, neocortex, and caudate/putamen. Two days of male exposure significantly increased the number of BrdU-labeled cells in the amygdala and hypothalamus in comparison to social isolation. Three weeks later, group differences in BrdU labeling generally persisted in the amygdala, whereas in the hypothalamus, the male-exposed animals had more BrdU-labeled cells than did the female-exposed animals. In the SVZ, 2 days of social isolation increased the number of BrdU-labeled cells compared to female exposure, but this difference was no longer present 3 weeks later. We have also found that the vast majority of the BrdU-labeled cells contained a neuronal marker, indicating neuronal phenotypes. Finally, group differences in the number of cells undergoing apoptosis were subtle and did not seem to account for the observed differences in BrdU labeling. Together, our data indicate that social environment affects neuron proliferation in a stimulus- and site-specific manner in adult female prairie voles.
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INTRODUCTION

Adult neurogenesis has been documented in several vertebrate species, including birds (Brown et al., 1993), rodents (Kaplan and Hinds, 1977; Luskin and Boone, 1994; Huang et al., 1998; Kempermann et al., 1998; Ormerod and Galea, 2001), nonhuman primates (Gould et al., 1999b), and humans (Eriksson et al., 1998). In the dentate gyrus (DG) of the hippocampus, cells proliferate in the subgranular zone and migrate into the granule cell layer where the majority develop into neurons (Cameron et al., 1993; Kuhn et al., 1996; Gould et al., 1997). In the subventricular zone (SVZ), cells proliferate and then migrate along the rostral migratory stream (RMS) to the olfactory bulb (Menezes et al., 1995; Peretto et al., 1999) where they disperse and differentiate into granule or periglomerular cells in the main olfactory bulb (Luskin, 1993; Peretto et al., 1999) or into granule cells in the accessory olfactory bulb (Bonfanti et al., 1997; Peretto et al., 1999). Recently, newly proliferated cells have been visualized in other brain regions, including the neocortex, preoptic area, central gray, thalamus, and hypothalamus (Huang et al., 1998; Gould et al., 1999b; Pencea et al., 2001). The origin of these cells is still unknown, although it has been suggested that the new neurons in the neocortex may migrate from the SVZ (Gould et al., 1999b).

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Although neurogenesis occurs continuously throughout adulthood, the rate of proliferation and the fate of the new cells may be affected by exogenous factors. For example, an enriched environment promotes the survival of cells undergoing proliferation in the DG of mice and rats (Kempermann et al., 1997, 1998; Nilsson et al., 1999). A short-day photoperiod enhances the number of proliferating cells in the DG, cingulate cortex, and hypothalamus of male hamsters (Huang et al., 1998). The rate of cell proliferation in the DG fluctuates in association with breeding season in female meadow voles (Galea and McEwen, 1999), and this effect may be attributable to changes in estrogen levels across seasons (Ormerod and Galea, 2001). In female prairie voles, male exposure induces an increase in cell proliferation in the SVZ (Smith et al., 2001). Finally, exposure to an unfamiliar male decreases the number of proliferating cells in the DG of male tree shrews, possibly due to psychosocial stress (Gould et al., 1997). Together, these data indicate that an animal's environment affects adult neurogenesis, which in turn, may impact behavioral and cognitive functions (Gould et al., 1999a; van Praag et al., 1999; Shors et al., 2001).

The prairie vole (*Microtus ochrogaster*) has been characterized as highly social and appears to form selective attachments (Carter and Getz, 1993; Getz and Carter, 1996). Previous studies have demonstrated that, unlike rats, female prairie voles lack an estrous cycle and are induced into behavioral estrus by 24–48 h of exposure to a male or male-associated sensory cues (Cohen-Parsons and Carter, 1987). Such male-induced behavioral estrus is associated with profound hormonal changes, including a rise in serum estrogen and an increase in estrogen receptors in the brain (Dluzen and Carter, 1979; Hnatczuk and Morrell, 1995; Smith et al., 2001). Therefore, the prairie vole provides an opportunity to examine the effects of environmental and endocrine changes on physiology and behavior.

In the present study, we examined the effects of social environment, specifically male exposure or social isolation, on adult neurogenesis in female prairie voles. We also identified the phenotype of the newly proliferated cells and investigated the rate of apoptosis in response to social environment. It was hypothesized that male exposure and social isolation would differentially affect adult neurogenesis in female prairie voles in a region-specific manner.

MATERIALS AND METHODS

Subjects

Subjects were sexually naive female prairie voles (*Microtus ochrogaste*r) that were offspring of the F3 generation of a laboratory-breeding colony. The voles were weaned at 21 days of age and housed in same-sex sibling pairs in plastic cages (29 × 18 × 13 cm) that contained cedar chip bedding. All cages were maintained under 14L:10D photoperiod with lights on at 0700 h. Temperature was kept at 21 ± 1°C. Animals were provided with food (rabbit chow) and water *ad libitum.* Female voles (85–135 days of age) used as subjects or stimulus animals were randomly assigned to treatment groups. Stimulus males for the male-exposure group were sexually experienced adult males from our colony.

BrdU Injections

To label proliferating cells, female subjects were injected with a cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU; Sigma: St. Louis, MO). Injections began 24 h after placement into treatment condition and continued at 6-h intervals during the second 24 h of treatment (total of four injections per animal). BrdU injections were given intraperitoneally (i.p.; 50 μg/g body weight) in 0.9% NaCl and 0.007N NaOH, as described previously (Smith et al., 2001).

Experiment 1

Female subjects were randomly assigned to one of three treatment groups: housed with an unfamiliar male (male exposure), housed with an unfamiliar female (female exposure), or housed alone (isolation). Subjects in each treatment group were further divided into two subgroups sacrificed at either 2 days or 3 weeks following environmental manipulation. The animals remained in their respective social environments until time of sacrifice. During the first 48 h of treatment, the male-exposure group was videotaped to verify copulation; subjects that did not mate were excluded from the study. At 3 weeks, the male-exposure group had successfully produced their first litter. Litter births occurred over the span of 2 days, and subjects were sacrificed 3 days following litter birth. Females from the female-exposure and isolation groups were sacrificed concurrently with females from the male-exposure group to control for time of sacrifice. The purpose of the 3-week subgroups was to examine the effects of social environment on the survival of the newly added cells in comparison to the effects seen at two days. Concerning the 3-week male-exposure group, although pregnancy and parturition may have introduced additional effects on the new cells, we were interested in the long-term effects in a situation comparable to what may naturally occur in the animal's environment, so the animals were permitted to become pregnant and deliver the pups. Therefore, the resulting treatment groups included male-exposure (n = 7), female-exposure (n = 6), and isolation (n = 7) sacrificed at 2 days, as well as male-exposure (n = 6), female-exposure (n = 8), and isolation (n = 7) sacrificed at 3 weeks.

Brain Perfusion/Fixation

Subjects were anesthetized with sodium pentobarbital and perfused through the ascending aorta using 0.9% saline followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer solution (PBS; pH 7.4). Brains were harvested, post-fixed for 2 h in 4% paraformaldehyde, and then stored in 30% sucrose in PBS. Brains were then blocked on the coronal plane, caudal to the optic chaism. The rostral portion was cut into 40-μm sagittal sections with a vibratome. From those sections, the subventricular zone (SVZ), rostral migratory stream (RMS), and olfactory bulb were visualized. The caudal portion was cut into 40 μm coronal sections, allowing the dentate gyrus (DG), amygdala, cingulate cortex, hypothalamus, and caudal portions of the caudate/putamen to be visualized. All of the brain sections were stored in 0.1 *M* PBS with 1% sodium azide until processing either for peroxidase BrdU immunostaining or for double or triple fluorescence immunolabeling.

BrdU Immunocytochemistry

Floating brain sections at 120-μm intervals were processed for BrdU immunostaining, as previously described (Smith et al., 2001). Sections were treated with 2 *N* HCl for 30 min at 60°C and then with 0.1 *M* borate buffer at room temperature for 25 min. After rinsing in 0.1 *M* PBS, sections were incubated in 0.3% hydrogen peroxide and 10% methanol in 0.1 *M* PBS for 15 min; 0.5% Triton X-100 in 0.1 *M* PBS with 10% normal goat serum (blocking serum) for 60 min; and rat anti-BrdU monoclonal antibody (1:1,000; Accurate: Westbury, NY) in blocking serum at 4°C overnight. Sections were then rinsed and incubated in biotinylated goat anti-rat IgG (1:200; Jackson ImmunoResearch: West Grove, PA) in blocking serum for 2 h at room temperature. Thereafter, sections were incubated in ABC Vector Elite in 0.1 *M* PBS for 90 min and immunoreactivity was revealed using 3′-diaminobenzidine (DAB; Sigma). Controls included processing brain sections without the primary antibody and processing brain sections from animals that did not receive BrdU injections; in either case, BrdU immunoreactive staining was not detected. In addition, to reduce variability in the background and to standardize the staining, sections from all subjects were processed concurrently for BrdU immunostaining.

Double or Triple Fluorescence Immunolabeling

To determine the phenotype of the BrdU-labeled cells, floating sections at 120-μm intervals were processed for BrdU and TuJ1 fluorescence double labeling or fluorescence triple labeling with BrdU, GFAP, and either MAP-2 or NeuN. TuJ1 is a mouse monoclonal IgG that recognizes a neuron-specific class III β-tubulin. This tubulin is considered to be the earliest marker for cells that have begun to differentiate into neurons (Alexander et al., 1991; Kameda et al., 1993). GFAP is a goat polyclonal IgG that recognizes glial fibrillary acidic protein found in astroglia and has been previously used to identify colocalized BrdU and glial cells (Nilsson et al., 1999; Magavi et al., 2000). MAP-2 is a protein associated with the dendrites and cytoplasm of mature neurons (Johnson and Jope, 1992), and NeuN is a protein that first appears after the cell has differentiated into a mature neuron (Mullen et al., 1992). TuJ1, MAP-2, and NeuN antibodies have all been shown to successfully label neurons that have undergone proliferation in the adult mammalian brain (Eriksson et al., 1998; Gould et al., 1999b; Magavi et al., 2000; Smith et al., 2001).

For BrdU/TuJ1 double labeling, sections from the 2-day subgroups were first processed for BrdU immunocytochemistry but were incubated in rat anti-BrdU (1:200; Accurate) in 0.1 *M* PBS with 0.1% Triton X-100 (PBT) at 4°C overnight and in rhodamine-conjugated goat anti-rat IgG (1:100; Jackson Immuno.) for 2 h at room temperature. Sections were then rinsed in PBT, blocked in 10% normal rabbit serum in PBT for 60 min, and incubated in mouse anti-TuJ1 (1:500; Covance: Richmond, CA) at 4°C overnight, followed by 60 min at room temperature. Thereafter, sections were rinsed in PBT and incubated in fluorescein-conjugated rabbit anti-mouse IgG (1:200; Jackson Immuno.) for 2 h at room temperature.

For BrdU/GFAP/MAP-2 or BrdU/GFAP/NeuN triple labeling, sections from the 3-week subgroups were first labeled for BrdU and blocked with 10% normal donkey serum, as described above, but Texas red-conjugated donkey anti-rat IgG was used as the secondary antibody. Sections were then blocked in 10% normal donkey serum in PBT for 60 min and incubated in goat anti-glial fibrillary acidic protein (GFAP, 1:1000; Santa Cruz: Santa Cruz, CA) in PBT at 4°C overnight, followed by 60 min at room temperature. Thereafter, sections were rinsed and incubated in cy5-conjugated donkey anti-goat IgG (1:100, Jackson Immuno.) for 2 h at room temperature. After rinsing in PBT and blocking in 10% normal donkey serum for 60 min, sections were incubated in either mouse anti-MAP-2 (1:500; Sigma) or mouse anti-NeuN (1:100; Chemicon: Temecula, CA) in PBT at 4°C overnight, followed by 60 min at room temperature. Thereafter, sections were rinsed in PBT and incubated in fluorescein-conjugated donkey anti-mouse IgG (1:200; Jackson Immuno.) for 2 h at room temperature. Finally, sections were rinsed in 0.1 *M* PBS, mounted using SlowFade (Molecular Probes: Eugene, OR) and coverslipped. The immunoflourescent labeling was then visualized using confocal microscopy. Controls included processing the secondary antibodies alone to verify background staining, processing the primary with the secondary antibodies to verify laser-specific excitation, and using sequential scans with triple labeling to avoid crosstalk between channels.

Data Quantification and Analysis

All slides were coded to disguise group identity until after every section had been analyzed. For peroxidase BrdU immunostaining, BrdU-positive ($BrdU^+$) cells were exam-

ined in the dentate gyrus of the hippocampus (DG), central, medial, and cortical nuclei of the amygdala, arcuate and ventromedial nuclei of the hypothalamus, posterior cingulate cortex, and caudate/putamen on the coronal sections for all groups. On the sagittal sections, BrdU-labeled cells were examined in the SVZ and RMS for the 2-day subgroups and in the granule cell layer of the main olfactory bulb (MOB) and in the accessory olfactory bulb (AOB) for the 3-week subgroups. Because SVZ-derived cells migrate along the RMS into the olfactory bulb (Luskin, 1993; Menezes et al., 1995), BrdU-labeled cells were absent in the SVZ at 3 weeks and, instead, were distributed throughout the olfactory bulb at this time.

BrdU-labeled cells were visualized under 40× magnification using a Zeiss AxioskopII microscope, and images were captured using a computerized image program (NIH Image 1.60). In the SVZ, BrdU-labeled cells were counted in two microscope fields (0.037 mm^2 each) per section, beginning rostral to the lateral ventricle and extending down the superior portion of the RMS. In the MOB, BrdU-labeled cells were counted in six microscope fields (0.037 mm^2 each) of the granule cell layer per section. BrdU-labeled cells were counted over the entire area of the AOB and bilaterally in the hilus and granule cell layers of the DG [corresponding to Plates 29–32 in Paxinos and Watson (1998)]. For the above-mentioned areas, 6–10 sections per area per animal were examined, and all the sections were carefully matched anatomically between animals. Furthermore, BrdU-labeled cells in the central, medial, and cortical nuclei of the amygdala [Plates 28–29 in Paxinos and Watson (1998)], arcuate, and ventromedial nuclei of the hypothalamus [Plates 30–32 in Paxinos and Watson (1998)], posterior cingulate cortex [Plates 23–24 in Paxinos and Watson (1998)] and caudate/putamen [Plates 29–31 in Paxinos and Watson (1998)] were examined bilaterally on three sections per animal with sections matched between animals. Cell counts were averaged over the number of sections for each brain area, and means were used for data analysis. Treatment effects for the number of BrdU-labeled cells at each time point were analyzed by a one-way analysis of variance (ANOVA), followed by a Student-Newman-Keul's post hoc (SNK) test.

BrdU and TuJ1 labeled cells were quantified in the DG and SVZ from the 2-day subgroups. BrdU/GFAP/MAP-2 or BrdU/GFAP/NeuN labeled cells were examined in the DG and olfactory bulb from the 3-week subgroups. Cells were visualized under 63× magnification using a Bio-Rad 1024 confocal microscope. For each area, at least 40 cells were counted from two sections per animal. Individual cells stained for BrdU/TuJ1, BrdU/MAP-2, BrdU/NeuN, BrdU/GFAP, or BrdU-only were counted. Percentages were calculated for the individual subject's number of double-labeled cells divided by the corresponding subject's total number of BrdU-labeled cells. Group differences in the percentage of BrdU-labeled cells containing a neuronal marker (TuJ1, MAP-2, or NeuN) or a glial marker (GFAP) were analyzed at each time point by a one-way ANOVA, followed by a SNK test. None of the BrdU-labeled cells were found to contain both neuronal (TuJ1, MAP2, or NeuN) and glial (GFAP) markers. The presence of double-labeled cells was also verified in the amygdala and hypothalamus. However, due to limited brain sections, quantitative data were not obtained from those brain regions.

Experiment 2

As data from Experiment 1 indicated that manipulation of social environment significantly alters the number of BrdU-labeled cells, we further tested whether changes in the number of BrdU-labeled cells were due to group differences in the rate of apoptosis at 2 days or 3 weeks. For the 2-day subgroups, female prairie voles were assigned to one of three treatment groups: male-exposure ($n = 6$), female-exposure ($n = 6$), or isolation ($n = 5$); all animals received four BrdU injections according to the paradigm described in Experiment 1. Forty-eight hours following the initiation of treatment, subjects were decapitated, and brains were removed, blocked on the coronal plane caudal to the optic chiasm, and frozen on dry ice. The rostral portion was cut into 20-μm sagittal sections on a cryostat, while the caudal portion was cut into 20-μm coronal sections. Sagittal sections through the SVZ, RMS, and OB and coronal sections through the hippocampus [Plates 29–32 in Paxinos and Watson (1998)] were thaw mounted onto Superfrost/plus slides (Fisher: Springfield, NJ) at 60-μm intervals and were stored at −80°C before processing for apoptosis labeling. For the 3-week subgroups, floating sections at 120-μm intervals from Experiment 1 were used.

Apoptosis Labeling

To evaluate DNA fragmentation resulting from apoptosis, floating and slide-mounted sections were processed for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Kit S7101, Intergen: Purchase, NY). This method has been extensively used in rodents including prairie voles (Thomaidou et al., 1997; Hastings et al., 1999; Biebl et al., 2000; Zhu et al., 2000). Briefly, sections were fixed in 4% paraformaldehyde in PBS (0.05 *M* phosphate sodium, 0.2 *M* NaCl, pH 7.4) at 4°C for 15 min, postfixed in precooled ethanol:acetic acid (2:1) at −20°C for 5 min, and incubated in proteinase K (5 μg/mL) for 15 min at room temperature. Three percent H_2O_2 was used to quench endogenous peroxidase activity. Afterwards, sections were incubated in working strength TdT enzyme for 2 h at 37°C in a humidified chamber, followed by working strength stop/wash buffer for 10 min. Sections were then incubated in antidigoxigen peroxidase conjugate in a humidified chamber at room temperature for 60 min, followed by working strength peroxidase substrate at room temperature until staining was visualized. The floating sections for the 3-week subgroups were then mounted. All sections were counterstained with 0.25% methyl green at room temperature for 5 min, washed in 100% 1-butanol, and dehydrated in xylene. Slides were coverslipped using Permount

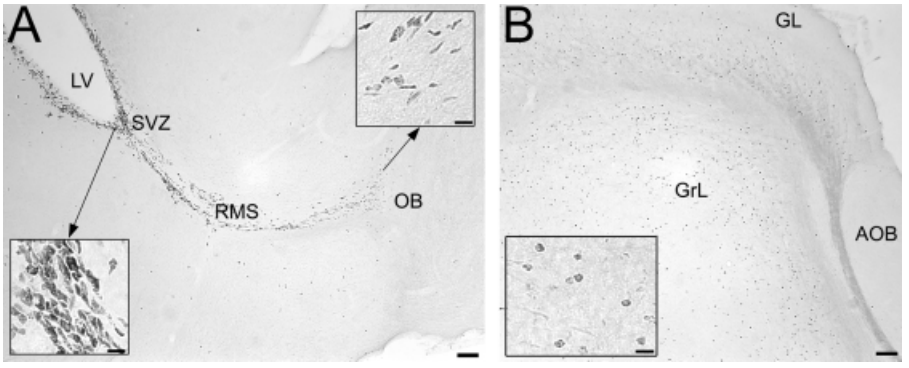

Figure 1 Photomicrographs of BrdU-labeled cells in the subventricular zone (SVZ), rostral migratory stream (RMS), and olfactory bulb (OB). (A) At 2 days, the majority of the BrdU-labeled cells are found in the SVZ and RMS but fewer are present in the OB. Scale bar = 100 μm. The bottom left insert displays densely packed cells labeled for BrdU in the SVZ (Scale bar = 10 μm) while the top right insert displays scattered cells labeled for BrdU in the OB (Scale bar = 10 μm). (B) At 3 weeks, BrdU-labeled cells are distributed throughout the cell layers of the main olfactory bulb, and a few are present in the accessory olfactory bulb (AOB). Scale bar = 100 μm. The insert displays BrdU-labeled cells in the granule cell layer (GrL) of the main olfactory bulb (Scale bar = 10 μm). LV: lateral ventricle; GL: glomerular layer.

(Fisher). Controls included first digesting sections with DNase prior to TdT treatment (positive control) and processing sections without the TdT enzyme treatment (negative control); both revealed no labeling.

Coded slides were examined by two experimenters using a dual-view microscope (Olympus BX50). TUNEL-labeled cells in the DG and SVZ for the 2-day subgroups and in the DG and OB for the 3-week subgroups were counted under 40× magnification. Cell counts were performed on three to six sections per area for each animal with sections matched across subjects. The mean number of TUNEL-labeled cells per section was calculated, and group differences were analyzed by a one-way ANOVA, followed by a SNK test.

RESULTS

BrdU Immunoreactive Labeling

BrdU immunocytochemistry produced dense nuclear staining of cells in specific areas of the vole brain. Manipulation of social environment altered the number of BrdU-positive ($BrdU^+$) cells in a stimulus- and region-specific manner. In the SVZ, dense clusters of $BrdU^+$ cells were found in subjects from the 2-day subgroups; densely packed $BrdU^+$ cells were also found in the intermediate pathway of the RMS, but few cells were present in the olfactory bulb [Fig. 1(A)]. After 2 days of treatment, the isolation group had significantly more $BrdU^+$ cells in the SVZ than did the female-exposure group, $F(2, 15) = 3.713$, $p < 0.05$ [Fig. 2(A)]; a similar increase in BrdU labeling was found in the male-exposure group, but this did not reach statistical significance. After 3 weeks of treatment, $BrdU^+$ cells were absent in the SVZ and RMS and, instead, were distributed through-

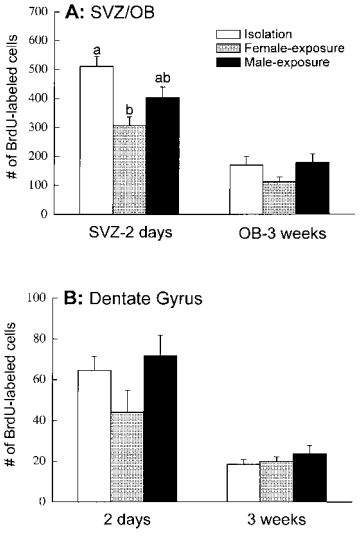

Figure 2 The effects of social environment on the mean number of BrdU-labeled cells in female prairie voles. (A) In the subventricular zone (SVZ), the isolation group at 2 days had significantly more BrdU-labeled cells than did the female-exposure group. At 3 weeks, the labeled cells had migrated into the olfactory bulb (OB) where no treatment effects were detected. (B) In the dentate gyrus, statistically significant differences were not found at either time point, although a trend does appear to exist at 2 days. Alphabetical letters represent the results of the post hoc test. Error bars indicate standard error of the mean.

out the olfactory bulb [Fig. 1(B)]. Most of the $BrdU^+$ cells were found in the granule cell layer of the MOB, and a few were present in the AOB. No group differences were found in the number of $BrdU^+$ cells either in the MOB [Fig. 2(A)] or AOB [isolation: 7.7 ± 1.4; female-exposure: 7.6 ± 0.6; male-exposure: 9.3 ± 0.7). In the DG, manipulation of social environment did not significantly influence the number of $BrdU^+$ cells after either 2 days or 3 weeks of treatment [Fig. 2(B)].

$BrdU^+$ cells were also visualized in other brain areas, in addition to the SVZ, RMS, OB, and DG. We quantified the number of $BrdU^+$ cells in the amygdala (central, medial, and cortical nuclei), hypothalamus (arcuate nucleus and VMH), posterior cingulate cortex, and caudate/putamen. Manipulation of social environment altered the number of $BrdU^+$ cells in a stimulus- and site-specific manner. In the amygdala [Fig. 3(A)–(C) and 4(A)] after 2 days of treatment, the male-exposure group had significantly more $BrdU^+$ cells than did the isolation group, $F(2, 10) = 5.39$, $p < 0.05$. Specifically, in the central nucleus, the male-exposure group displayed a larger number of $BrdU^+$ cells than did the isolation group, $F(2, 10) = 6.34$, $p < 0.05$, but such differences were not detected in the cortical or medial nuclei at this time point. At 3weeks, a similar pattern was found as the male-exposure group had significantly more $BrdU^+$ cells than did both the isolation and female-exposure groups, $F(2, 18) = 5.79$, $p < 0.05$. When examining the specific amgdaloid nuclei, the medial and cortical, but not the central, nuclei displayed group differences. In the medial nucleus, the male-exposure group had significantly more $BrdU^+$ cells than did the isolation group, $F(2, 18) = 3.62$, $p < 0.05$. In the cortical nucleus, the male-exposure group displayed a larger number of $BrdU^+$ cells than did both the isolation and female-exposure groups, $F(2, 18) = 7.60$, $p < 0.05$. A similar pattern was found in the hypothalamus [Fig. 3(D)–(F) and 4(B)] with male exposure increasing the number of $BrdU^+$ cells at 2 days compared to social isolation, $F(2, 11) = 4.38$, $p < 0.05$, but at 3 weeks, the male-exposure group displayed a larger number of $BrdU^+$ cells than did the female-exposure group, $F(2, 18) = 8.28$, $p < 0.05$. Finally, in the cingulate cortex [Fig. 3(G)–(I) and 4(C) and caudate/putamen (Fig.

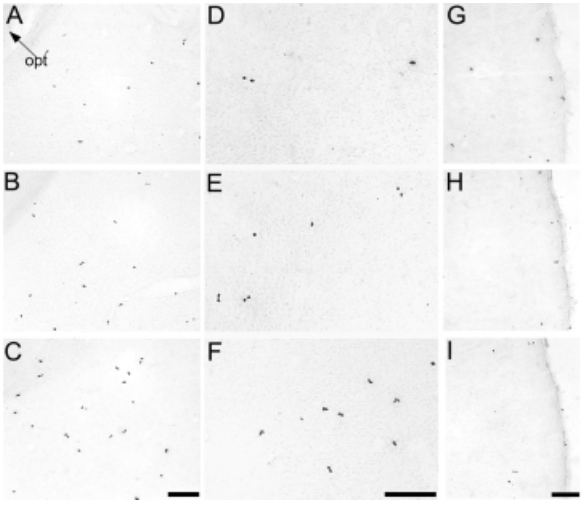

Figure 3 Photomicrographs of BrdU-labeled cells at 2 days following treatment in the (A–C) amygdala, (D–F) hypothalamus and (G–I) cingulate cortex of female prairie voles. In the amygdala, the isolation group (A) had significantly less BrdU-labeled cells than did the male-exposure group (C); the female-exposure group (B) did not differ significantly from either other group. In the hypothalamus, the isolation group (D) had significantly less BrdU-labeled cells than did the male-exposure group (F); the female-exposure group (E) did not differ from either other group. In the cingulate cortex, the isolation (G), female-exposure (H), and male-exposure (I) groups did not differ in the number of BrdU-labeled cells. Scale bars = 100 μm.

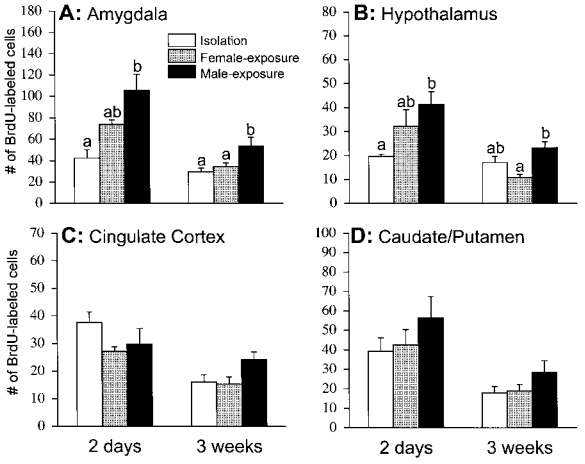

Figure 4 The effects of social environment on the mean number of BrdU-labeled cells in female prairie voles. (A) In the amygdala, at 2 days, the male-exposure group had significantly more BrdU-labeled cells than did the isolation group, but the female-exposure group did not differ from either other group. At 3 weeks, the male-exposure group displayed more BrdU-labeled cells than did both the isolation and female-exposure groups. (B) In the hypothalamus at 2 days, the isolation group had less BrdU-labeled cells than did male-exposure group, but the female-exposure group did not differ from either. At 3 weeks, the male-exposure group had more BrdU-labeled cells than did the female-exposure group, but the isolation group did not differ from either. In the cingulate cortex (C) and caudate/putamen (D), group differences were not found at 2 days or at 3 weeks. Alphabetical letters represent the results of the post hoc test. Error bars indicate standard error of the mean.

Table 1 Percentage of BrdU$^+$ Cells Double-Labeled with a Neuronal or Glial Marker

		2 Days	3 Weeks		
Brain Area	Treatment	BrdU/TuJ1	BrdU/MAP-2	BrdU/NeuN	BrdU/GFAP
DG	Isolation	77	87	85	3
	Control	75	86	85	1.4
	Mating	64	84	100	0
SVZ	Isolation	30	—	—	—
	Control	43	—	—	—
	Mating	27	—	—	—
OB	Isolation	—	92	89	0
	Control	—	90	91	0
	Mating	—	86	96	0.3

4D) no group differences in the number of BrdU$^+$ cells were detected at either time point.

Phenotype of the BrdU-Labeled Cells

The percentages of the BrdU$^+$ cells in the DG, SVZ, and OB containing a neuronal or a glial maker are summarized in Table 1. For the 2-day subgroups, the majority (72%) of the BrdU$^+$ cells in the DG were also TuJ1 positive [Fig. 5(A)]. Fewer (33%) BrdU$^+$ cells were colocalized with TuJ1 in the SVZ [Fig. 5(B)]. In both areas, no group differences were detected in the percentage of the BrdU$^+$ cells containing TuJ1 staining. Cells double-labeled with BrdU and TuJ1 were also found in the amygdala [Fig. 5(C)] and hypothalamus [Fig. 5(D)], although quantitative data were not obtained. For the 3-week subgroups, in the DG, the vast majority of the BrdU$^+$ cells were colocalized with either MAP-2 [86%; Fig. 6(A)] or NeuN (90%), whereas a minimal percentage (1.5%) were colocalized with GFAP. Similarly in the olfactory bulb, the majority of the BrdU$^+$ cells were colocalized with either MAP-2 (90%) or NeuN [92%; Fig. 6(B)], and a minor percentage (0.1%) were colocalized with GFAP. No group differences were found for cells double-labeled with BrdU and either MAP-2, NeuN, or GFAP in any of these brain areas.

Apoptosis Labeling

The TUNEL labeling resulted in dark-brown nuclear staining (Fig. 7). TUNEL-positive (TUNEL$^+$) cells were found in the SVZ and RMS [Fig. 7(A)], granule cell layer of the OB [Fig. 7(B)], and granule layer [Fig. 7(C)] and hilus of the DG. After 2 days of treatment, group differences were not found in the SVZ [Fig. 8(A)], but in the DG [Fig. 8(B)], the male-exposure and isolation groups had more TUNEL$^+$ cells than the female-exposure group, $F(2, 14) = 4.76$, $p < 0.05$. No group differences in the number of TUNEL$^+$ cells were found in the OB [Fig. 8(A)] or DG [Fig. 8(B)] after 3 weeks of treatment. We also observed scattered TUNEL$^+$ cells in several other brain areas, such as the hypothalamus [Fig. 7(D)], amygdala, and neocortex, although limitations in the number of brain sections prevented us from reliably quantifying those cells for comparison among treatment groups.

DISCUSSION

Previous studies have demonstrated that environmental factors, such as environmental complexity (Kempermann et al., 1997, 1998; Nilsson et al., 1999), photoperiod (Huang et al., 1998), seasonal changes (Galea and McEwen, 1999), and psychosocial stress (Gould et al., 1997), affect adult neurogenesis in the mammalian brain. In the present study, we found that male exposure or social isolation altered neurogenesis in adult female prairie voles in a stimulus- and region-specific manner. Differential effects were induced after either short-term (e.g., SVZ) or long-term (e.g., hypothalamus) exposure depending on the brain region. In addition, group differences in the number of cells undergoing apoptosis were subtle, suggesting that social environment most likely affects the proliferation of cells in the female prairie vole brain.

In the present study, BrdU was injected at a concentration of 50 mg/kg, a dosage that has been shown to be nontoxic (Miller and Nowakowski, 1988) and has been commonly used for most studies of adult neurogenesis (Huang et al., 1998; Kempermann et al., 1998; Nilsson et al., 1999; Cameron and McKay, 2001; Smith et al., 2001). A recent report suggests that a higher dosage of BrdU (300 mg/kg) may be a better quantitative marker of proliferating cells in the DG of rats, as lower dosages (e.g., 50 mg/kg) only

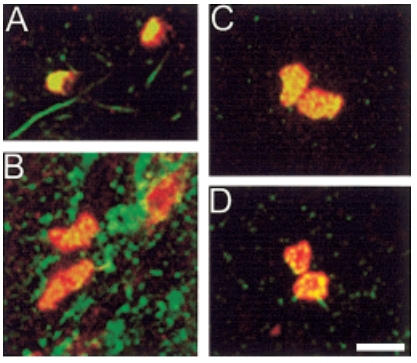

Figure 5 Confocal laser microscope images of cells colocalized (yellow) for BrdU (red) and TuJ1 (green) in the dentate gyrus (A), subventricular zone (B), central amygdala (C), and ventromedial hypothalamus (D) of female prairie voles after 2 days of treatment. Scale bar = 5 μm.

label a fraction of the S-phase cells (Cameron and McKay, 2001). However, our injection schedule (four injections at 6-h intervals during a 24-h period) was previously shown to label a large number of proliferating cells in prairie voles (Smith et al., 2001). Furthermore, subjects in all groups received the same dosage of BrdU, and thus proliferating cells labeled with BrdU should have been proportional across treatment groups, allowing for an accurate comparison.

New Cells in the Amygdala and Hypothalamus

Observations of $BrdU^+$ cells in brain areas other than the DG, SVZ, and OB have been reported in hamsters, rats, and nonhuman primates (Huang et al., 1998; Gould et al., 1999b; Pencea et al., 2001). In the present study, $BrdU^+$ cells were found in the amygdala, hypothalamus, neocortex, and caudate/putamen of the prairie vole brain. An important finding is that the number of $BrdU^+$ cells was affected by social environment. In both the amygdala and hypothalamus, for example, 2 days of male exposure significantly increased the number of $BrdU^+$ cells in comparison to social isolation. At 3 weeks, the same general pattern persisted in the amygdala, whereas in the hypothalamus, male exposure increased the number of $BrdU^+$ cells compared to female exposure. Although we did not quantify the number of double-labeled cells, there were some $BrdU^+$ cells in the amygdala and hypothalamus that displayed a neuronal phenotype. These data provide evidence to support our hypothesis that social environment influences the newly proliferated neurons in the adult female prairie vole brain.

Several factors associated with social experience may have contributed to the group differences in BrdU labeling. During 48 h of male exposure, female prairie voles experience an increase in serum estrogen; their estrogen levels are elevated within 18 h following male exposure and persist for at least 4–5 days (Carter et al., 1986; Cohen-Parsons and Carter, 1987). Because elevated estrogen is a prerequisite for voles to display estrous behavior (Dluzen and Carter, 1979), mating was used as a behavioral index to ensure that all subjects in the male-exposure group experienced an increase in estrogen. In recent studies, estrogen was found to enhance BrdU labeling in the SVZ of female prairie voles (Smith et al., 2001) and in the DG of female meadow voles (Ormerod and Galea, 2001). If these findings can be generalized, we would expect the elevation of estrogen following male exposure to be responsible for the increased BrdU labeling in the amygdala and hypothalamus. This no-

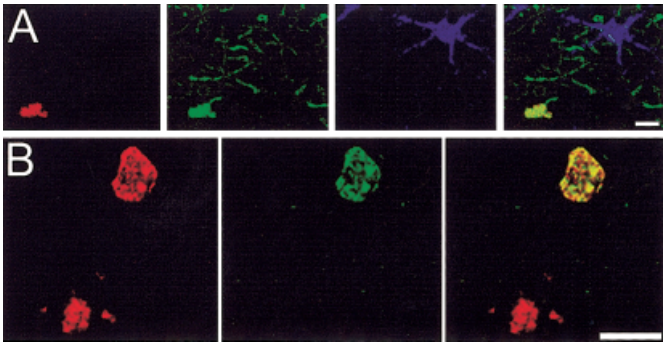

Figure 6 Confocal laser microscope images of cells stained for BrdU, MAP-2, NeuN, and/or GFAP in female prairie voles. (A) In the dentate gyrus, cells display staining for BrdU (red), MAP-2 (green), GFAP (blue), and all three markers (right panel). The BrdU and MAP-2 colocalized cell displays a yellow image (right panel). (B) In the olfactory bulb, a single BrdU (red) labeled cell and a BrdU and NeuN (green) double-labeled cell (yellow) are shown. Scale bar = 5 μm.

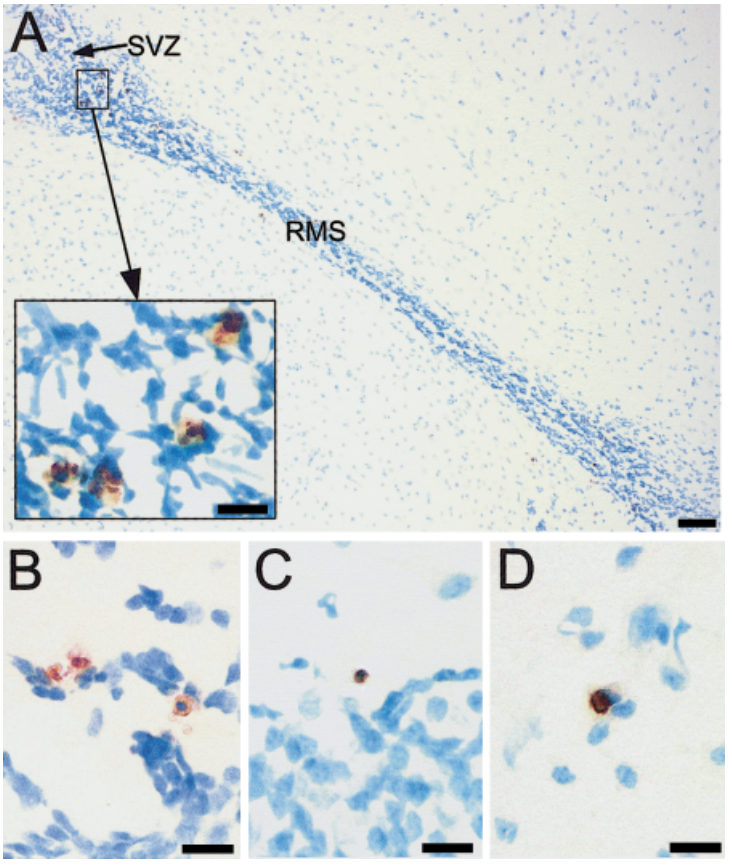

Figure 7 (A) Photomicrographs of TUNEL-labeled cells (brown) in the subventricular zone (SVZ) and rostral migratory stream (RMS) of a female prairie vole. Scale bar = 50 μm. The insert displays TUNEL-labeled cells in the SVZ (scale bar = 10 μm). TUNEL-labeled cells were also found in the (B) granule cell layer of the olfactory bulb, (C) granule cell layer of the dentate gyrus, and (D) ventromedial hypothalamus. Scale bars = 10 μm.

tion is supported by the fact that, in the DG of female rats, BrdU labeling fluctuates during the estrous cycle, and ovariectomy decreases, whereas estrogen replacement restores, the number of proliferating cells (Tanapat et al., 1999).

Prairie voles are a social species (Getz and Carter, 1996) that display high levels of affiliative behavior (Dewsbury, 1987; Shapiro and Dewsbury, 1990), so our social isolation paradigm was expected to provide the voles with a stressful environment. Indeed, in female prairie voles, social isolation significantly elevates serum corticosterone, and in contrast, exposure to an unfamiliar female does not alter, whereas exposure to a male decreases, serum corticosterone levels relative to baseline levels (DeVries et al., 1995; Kim and Kirkpatrick, 1996). Therefore, the stress-induced elevation in corticosterone may have been responsible for the decreased BrdU labeling in the amygdala and hypothalamus of the isolated female prairie voles. This stress-induced decrease in the number of new cells is similar to previous studies that have examined neurogenesis in the DG of other mammals. In tree shrews, for example, social stress decreases cell proliferation (Gould et al., 1997), and in rats, treatment with corticosterone (Cameron et al., 1998) or hydrocortisone (Bohn, 1980) decreases, whereas adrenalectomy (Cameron and Gould, 1994) increases, the number of newly proliferated cells.

At present, the underlying regulatory mechanisms of hormones on neurogenesis are unknown, although some speculations may be drawn. For instance, in rats, estrogen upregulates BDNF expression in the hippocampus (Singh et al., 1995; Sohrabji et al., 1995; Gibbs, 1998). In contrast, acute or chronic stress downregulates BDNF expression in the hippocampus and in several hypothalamic brain regions via corticosterone-mediated mechanisms (Smith et al., 1995; Schaaf et al., 1997, 1998). BDNF administration enhances the proliferation and survival of cells in the SVZ and olfactory bulb, as well as in some thalamic

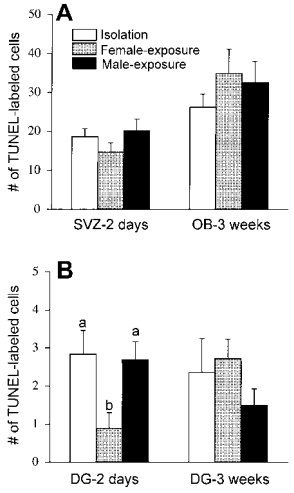

Figure 8 The effects of social environment on the mean number of TUNEL-labeled cells in female prairie voles. (A) Group differences in TUNEL labeling were not found in the subventricular zone (SVZ) at 2 days or in the olfactory bulb (OB) at 3 weeks. (B) In the dentate gyrus (DG), social isolation or male exposure for 2 days increased the number of TUNEL-labeled cells in comparison to female exposure; these treatment effects were not present 3 weeks later. Alphabetical letters represent the results of the post hoc test. Error bars indicate standard error of the mean.

and hypothalamic regions, in rats (Kirschenbaum and Goldman, 1995; Zigova et al., 1998; Pencea et al., 2001). In prairie voles, male exposure elevates circulating estrogen (Dluzen and Carter, 1979) and increases $BrdU^+$ cells in the amygdala and hypothalamus (present study), and estrogen treatment enhances BDNF expression in both brain regions (Liu et al., 2001). Finally, social isolation increases, whereas male exposure decreases, serum corticosterone levels (DeVries et al., 1995; Kim and Kirkpatrick, 1996). Together, these data suggest that altered estrogen and corticosterone may act via BDNF to regulate neurogenesis.

It is worth noting that in the amygdala, the pattern of group differences in BrdU labeling at 2 days persisted 3 weeks later. This finding is in distinct contrast to the isolation-induced transient increase in BrdU labeling in the SVZ in the present study and estrogen-induced transient increase in BrdU labeling in the DG of rats in a previous study (Tanapat et al., 1999). However, these data do support our hypothesis that the effects of social environment on adult neurogenesis are region specific. A simplistic explanation for our findings may be that the estrogen surge following male exposure or around parturition/postpartum (Dluzen and Carter, 1979; Carter et al., 1989; Smith et al., 2001) were responsible for the increased BrdU labeling in the amygdala and hypothalamus of the male-exposure group at 2 days and 3 weeks. Of course, the male-exposed animals at 3 weeks had undergone pregnancy and parturition, and many hormones (Carter et al., 1989; Neumann et al., 1998), in addition to estrogen, could have acted on the BrdU-labeled cells. Therefore, different mechanisms may have been regulating the cell proliferation and/or survival at 2 days than at 3 weeks. This speculation needs to be addressed in further studies.

Neurogenesis in the SVZ and DG

In the present study, social isolation for 48 h significantly increased the number of $BrdU^+$ cells in the SVZ in comparison to female exposure. Why did social isolation decrease BrdU labeling in the amygdala and hypothalamus but increase BrdU labeling in the SVZ? The explanation for such an unexpected increase is not obvious, and several possibilities may exist. First, two-thirds of the $BrdU^+$ cells in the SVZ did not display a neuronal phenotype (see Table 1). In a recent study, stress was found to induce cell proliferation in the non-neuronal olfactory epithelium of the adult mouse (Feron et al., 1999). It is possible, therefore, that the non-neuronal or undifferentiated population of $BrdU^+$ cells in the SVZ may account for the isolation-enhanced cell proliferation. Second, rather than social isolation increasing neurogenesis in the SVZ, the female exposure environment may have elicited an inhibitory effect, causing the number of $BrdU^+$ cells to decrease. This possibility can be ruled out because in the natural environment, it is common for individual voles to encounter the same or opposite sex strangers under high population densities (Getz et al., 1987) and in the laboratory, exposure to an unfamiliar female does not alter serum corticosterone levels in sexually naive female prairie voles (DeVries et al., 1995). Furthermore, female prairie voles exposed to a familiar female or to an unfamiliar female display similar levels of BrdU labeling in the SVZ (Smith et al., 2001), suggesting that exposure to an unfamiliar female does not negatively affect the newly proliferated cells.

A third possibility is that 2 days of social isolation provided a stressful stimulus, but with only site-specific effects: it reduced the number of newly added cells in the amygdala and hypothalamus but not in the SVZ or DG. The discrepancy that stress decreases neurogenesis in the DG of tree shrews and rats (Cameron and Gould, 1994; Gould et al., 1997; Cameron et al., 1998) but not in voles (present study) may be explained by the fact that prairie voles are a typical glucocorticoid-resistant animal that possesses a high

basal level of corticosterone and adrenal steroid receptors with low affinity and density in the DG (Taymans et al., 1997; Hastings et al., 1999). Therefore, the isolation-induced stress may not have negatively affected the addition of new cells in the DG, as it did in the amygdala and hypothalamus.

In a previous study of meadow voles, females exposed to a male for 48 h were found to have less $BrdU^+$ cells in the DG than females without male exposure; furthermore, an acute estrogen injection increased the number of $BrdU^+$ cells in the DG 4 h, but not 48 h, following the estrogen injection (Ormerod and Galea, 2001). In female prairie voles, however, exposure to a male for 48 h does not reduce BrdU labeling in the DG and SVZ (present study), and estrogen administration does elevate BrdU labeling moderately in the DG (M. Smith, unpublished data) and significantly in the SVZ (Smith et al., 2001). Why are there discrepancies between prairie and meadow voles for the effects of male exposure and estrogen on cell proliferation? Different paradigms incorporating different amounts and schedules of BrdU injections and estrogen treatment might contribute to these discrepancies. In addition, the two species show remarkable differences in life strategy; prairie voles are social and monogamous, whereas meadow voles are nonsocial and promiscuous (Dewsbury, 1987). Prior studies have shown that mating induces social attachment in monogamous, but not promiscuous, voles (Insel and Hulihan, 1995; Insel et al., 1995). In addition, administration of the neuropeptide vasopressin facilitates social attachment in monogamous voles but does not alter affiliative behavior in promiscuous voles (Winslow et al., 1993; Young et al., 1999; Liu et al., 2001). Therefore, social environment and estrogen may differentially influence adult neurogenesis depending on the vole's distinct life strategy and social behavior. It will be essential to conduct a carefully controlled comparative study examining the effects of social environment and/or steroid hormones on neurogenesis in monogamous and promiscuous voles before a precise conclusion can be drawn.

Changes in the number of $BrdU^+$ cells may have resulted from altered cell proliferation, survival, or both. To determine the role of cell death in the regulation of neurogenesis, TUNEL staining was used to indicate the number of cells undergoing apoptosis, a method that has be extensively used in rodents, including prairie voles (Thomaidou et al., 1997; Hastings et al., 1999; Biebl et al., 2000; Zhu et al., 2000). A decrease in TUNEL labeling can be used as an index of cell survival. We predicted that if increased BrdU labeling in the SVZ was due to decreased cell death, a decrease in TUNEL labeling would be observed for the mating and social isolation groups. Our data, however, demonstrated that male exposure or social isolation for 2 days enhanced TUNEL labeling in the DG but had no effects in the SVZ. Considering the magnitude of the number of $BrdU^+$ cells, the effect on TUNEL labeling appears to be subtle. These data suggest that social environment alters BrdU labeling most likely by acting on cell proliferation, rather than cell death, in female prairie voles. It should be noted that these results may not be conclusive. First, the TUNEL assay is based on DNA fragmentation and may also account for cells undergoing necrosis (Charriaut-Marlangue and Ben-Ari, 1995). Second, apoptosis was only measured at two time points (2 days and 3 weeks) in our study. Because the clearance time for $TUNEL^+$ cells is about 2–3 h (Thomaidou et al., 1997), it is possible that cells that had undergone apoptosis were already cleared from the cellular environment before the TUNEL labeling was performed. Finally, although differences in BrdU labeling were primarily present in the amygdala and hypothalamus, we were unable to sufficiently examine these areas for TUNEL.

CONCLUSION

In summary, manipulation of social environment resulted in stimulus- and site-specific effects on the newly proliferated cells in the adult female prairie vole brain. Our data demonstrate that experience with a male for 2 days significantly enhances the number of $BrdU^+$ cells in the amygdala and hypothalamus in comparison to social isolation. This general pattern in BrdU labeling persisted 3 weeks later, indicating that social environment also exerts long-term effects on the newly proliferated cells. In addition, we found that isolation for 2 days enhances cell proliferation in the SVZ, and social environment appears to act on cell proliferation, rather than cell death. Finally, many newly proliferated cells display a neuronal phenotype.

It is interesting to note that the amygdala has been implicated in olfactory memory (Demas et al., 1997), pheromonal analysis (Meredith, 1998), sexual behavior (Dominguez et al., 2001), and fear conditioning (Fanselow and LeDoux, 1999). In voles, mating induces social attachment (Williams et al., 1992a; Winslow et al., 1993; Insel et al., 1995), and the amygdala plays a role in memory and social attachment formation (Williams et al., 1992b; Kirkpatrick et al., 1994; Demas et al., 1997; Wang et al., 1997). Therefore, the addition of new cells in the amygdala may be of particular importance because newly added cells

could contribute to enhanced learning and memory abilities, as suggested in the DG of mice and rats (Kempermann et al., 1997; van Praag et al., 1999; Shors et al., 2001).

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