Newly Proliferated Cells in the Adult Male Amygdala Are Affected by Gonadal Steroid Hormones

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ABSTRACT: **Gonadal steroid hormones play an important role in the proliferation, survival, and activation of neurons. The present study was performed to examine the effects of testosterone and its metabolites on newly proliferated cells in the amygdala of adult male meadow voles (*Microtus pennsylvanicus*). Treatment with testosterone propionate (TP) in castrated males resulted in plasma testosterone levels similar to males following mating. TP-treated males displayed a significant increase in the density of cells labeled with a cell proliferation marker (BrdU) in the amygdala. Treatment with estradiol benzoate (EB) exerted a similar effect as TP on the density of BrdU-labeled cells, whereas 5α-dihydrotestosterone (DHT) was ineffective. A larger proportion (≈44%) of the BrdU-labeled cells in the amygdala displayed a neuronal phenotype, and a lesser percentage (≈35%) displayed a glial progenitor phenotype; however, treatment effects were not found in either population of cells. Hormonal effects appeared to be site-specific as no group differences were found in the dentate gyrus of the hippocampus or ventromedial hypothalamus. Finally, a time course study indicated that BrdU-labeled cells in the amygdala are present as early as 30 min following an acute injection of BrdU. Together, these data suggest that gonadal steroid hormones influence the number of newly proliferated cells in the amygdala, most likely by acting through an estrogenic mechanism, and these effects may be exerted on locally proliferating progenitors within the amygdala.** © 2003 Wiley Periodicals, Inc. J Neurobiol 57: 257–269, 2003

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INTRODUCTION

Adult mammalian neurogenesis may be influenced by a variety of exogenous and endogenous factors. In the dentate gyrus of the hippocampus (DG) of rodents, the number of new cells increases in response to environmental complexity (Kempermann et al., 1997; Nilsson et al., 1999), hippocampal-dependent learning (Gould et al., 1999a), or wheel running (van Praag et al., 1999). In contrast, psychosocial stress leads to a decrease in the number of new cells in the DG of tree shrews (Gould et al., 1997). The mechanisms underlying these effects may be attributed to changes in the animal's hormonal and/or neurotransmitter levels. For example, the inhibitory effects of stress on neurogenesis may occur via adrenal steroids, such as glucocorticoids (Cameron and Gould, 1994; Fuchs et al., 2001), whereas the enhancement of new cells may be influenced by the presence of serotonin or brain-derived neurotrophic factor (BDNF) (Brezun and Daszuta, 1999, 2000; Pencea et al., 2001).

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Although cells undergoing neurogenesis in the adult mammalian brain have been primarily documented in the DG and subventricular zone (SVZ), several other brain regions have been identified to contain newly proliferated cells. These regions include the amygdala and hypothalamus of voles and hamsters (Huang et al., 1998; Fowler et al., 2002), neocortex and amygdala of primates (Gould et al., 1999b; Bedard et al., 2001), and striatum, septum, and thalamus of rats (Pencea et al., 2001). Because the amygdala is responsive to gonadal hormones (Insel, 1990; Coolen and Wood, 1999) and has been implicated in reproductive-associated behaviors, such as olfactory/pheromonal processing (Luiten et al., 1985; Meredith, 1991), social learning and memory (Kirkpatrick et al., 1994; Cahill et al., 1996; Demas et al., 1997) and copulatory actions (Harris and Sachs, 1975; Dominguez et al., 2001), this region is of particular interest for the study of the hormonal effects on neurogenesis.

Gonadal steroid hormones may exert influential effects on cognition, memory, and mood in mammals, including humans (Vermeulen, 1993; Wang et al., 1996; Wolf and Kirschbaum, 2002). On the cellular level, these hormones may play a role in the proliferation (Ormerod and Galea, 2001), survival (Leranth et al., 2000), and activation (Insel, 1990) of neurons. Interestingly, estrogen administration does produce an increase in the number of new cells in the DG of adult female rats and meadow voles (Tanapat et al., 1999; Ormerod and Galea, 2001) and in the SVZ of adult female prairie voles (Smith et al., 2001). In the present study, we examined the effects of the gonadal steroid hormone, testosterone, and then its metabolites, estrogen and 5α-dihydrotestosterone (DHT), on the addition of new cells in the amygdala of male meadow voles (*Microtus pennsylvanicus*). We also identified the neuronal or glial phenotype of the newly proliferated cells in the amygdala. Finally, a time course study was performed to assess whether cells in the amygdala proliferate locally.

MATERIALS AND METHODS

Subjects

Subjects were sexually naive adult male meadow voles (*M. pennsylvanicus*) 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 $\times$ 18 $\times$ 13 cm) that contained cedar chip bedding. All cages were maintained under 14:10 light/dark photoperiod with lights on at 0700. Temperature was kept at 21 $\pm$ 1°C. Animals were provided *ad libitum* with food (rabbit chow and sunflower seeds) and water. Subjects were randomly assigned to experimental groups.

Treatment Groups

In the first experiment, subjects (2–3 months old) were castrated, followed by a recovery period of 2–3 weeks. Thereafter, they were implanted with Silastic tubing (10 mm long, 2 mm i.d., 3.2 mm o.d.) filled with oil vehicle (n = 7) or testosterone propionate (TP; n = 7; 0.1 mg/μL sesame oil, $\approx$20 μL each). In the second experiment, subjects (3–5 months old) were castrated, allowed to recover for 2–3 weeks, and were then divided into four groups that received implants of Silastic tubing filled with either oil vehicle (control, n = 5), TP (n = 6; 0.1 mg/μL oil), DHT (n = 7; 0.1 mg/μL oil), or estradiol benzoate (EB; n = 6; $\approx$1.5 mg each). In both experiments, subjects received hormonal or control treatment until sacrifice, which occurred 72 h after tubing implantation. In the third experiment, intact male meadow voles (2–4 months old) that received no hormonal treatment were used as subjects to examine the presence of newly proliferated cells. Subjects were sacrificed at 30 min or 1, 6, or 24 h following an acute injection of 5-bromo-2′-deoxyuridine (BrdU; n = 3 for each time point).

Injections of BrdU

To label proliferating cells, subjects were injected with a cell proliferation marker, BrdU (Sigma, St. Louis, MO). In experiments 1 and 2, injections began 48 h following tubing implantation and continued at 6 h intervals during the next 24 h of treatment (total of four injections per animal). Injections of BrdU were given intraperitoneally (ip; 50 μg/g body weight) in 0.9% NaCl and 0.007 *N* NaOH, as described previously (Smith et al., 2001). In experiment 3, one injection of a higher concentration of BrdU (300 μg/g body weight) was given to maximally label the number of proliferating cells, as indicated in a recent report (Cameron and McKay, 2001), and the subjects were then sacrificed either 30 min or 1, 6, or 24 h later.

Brain Perfusion/Fixation

Subjects were anesthetized with sodium pentobarbital (0.1 mg/10 g body weight) 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, postfixed for 2 h in 4% paraformaldehyde, and then stored in 30% sucrose in PBS. Brains were cut into 40 μm coronal or sagittal sections on a microtome, and the sections were stored in 0.1 *M* PBS with 1% sodium azide until processing either for peroxidase BrdU immunostaining or for triple fluorescence immunolabeling.

BrdU Immunocytochemistry

Floating brain sections at 120 μm intervals were processed for BrdU immunostaining. 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 antirat 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). To reduce variability in the background and to standardize the staining, sections from all subjects in each experiment were processed concurrently for BrdU immunostaining. As previously shown (Fowler et al., 2002), controls included processing brain sections without the primary antibody and processing brain sections from animals that did not receive injections of BrdU; in either case, BrdU immunoreactive staining was not detected.

Triple Fluorescence Immunolabeling

To determine the phenotype of the BrdU-positive ($BrdU^+$) cells, floating sections at 120 μm intervals were processed for BrdU, TuJ1, and NG2 fluorescence triple labeling. 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). NG2 is a polyclonal IgG that recognizes NG2, an integral membrane proteoglycan expressed on glial progenitor cells (Nishiyama et al., 1995); this proteoglycan has been identified in cells that mature into astrocytes (Fidler et al., 1999; Dawson et al., 2000), oligodendrocytes (Butt and Berry, 2000; Mallon et al., 2002), and microglia (Chang et al., 2000; Jones et al., 2002). Both TuJ1 and NG2 have been used to identify the phenotype of newly proliferated cells in adult brains (Shihabuddin et al., 2000; Fowler et al., 2002).

To triple label, sections were rinsed, blocked with 10% normal donkey serum in 0.1% Triton X-100 and 0.1 *M* PBS, and incubated in rabbit anti-NG2 (1:150; Chemicon) at 4°C overnight. On day 2, the sections was rinsed and incubated in Cy5-conjugated donkey antirabbit IgG (1:100; Jackson ImmunoResearch) for 2 h. Next, the sections were rinsed, incubated in 10% normal goat serum in 0.1% Triton-0.1 *M* PBS, and then incubated in mouse anti-TuJ1 (1:500; Covance) at 4°C overnight. Following rinsing on day 3, the sections were incubated in Alexa-488- conjugated goat antimouse IgG (1:400; Molecular Probes) for 2 h. Then, they were processed for BrdU immunocytochemistry by incubating in normal donkey serum, rat anti-BrdU (1:200; Accurate) in 0.1 *M* PBS with 0.1% Triton X-100 at 4°C 36 h, and Texas Red-conjugated donkey antirat IgG (1:200; Jackson ImmunoResearch) for 2 h at room temperature. Sections were rinsed, mounted on slides with slowfade component A (Molecular Probes), and coverslipped.

Data Quantification and Analysis

All slides were coded to disguise group identity. For peroxidase BrdU immunostaining, $BrdU^+$ cells were examined and quantified bilaterally in the DG, central (CeA), medial (MeA), and cortical (CorA) nuclei of the amygdala, and ventromedial nucleus of the hypothalamus (VMH) on coronal sections for all groups in experiment 1 and 2. For experiment 3, $BrdU^+$ cells in the CeA, MeA, CorA, and VMH were examined on sagittal sections and quantified on coronal sections.

$BrdU^+$ cells were visualized under 40X magnification using a Zeiss AxioskopII microscope. $BrdU^+$ cells were counted in the hilus, granule, and molecular cell layers of the DG (corresponding to Plates 29–32 in Paxinos and Watson, 1998) for seven to eight sections per animal; groups did not differ in the number of sections counted. In the central, medial, and cortical nuclei of the amygdala (Plates 28–29 in Paxinos and Watson, 1998) and VMH (Plates 30–32 in Paxinos and Watson, 1998) three sections were counted per animal. Furthermore, the areas of these brain regions were measured using the NIH IMAGE program. The images were projected to a computer screen and the boundaries of each region were traced bilaterally. Because no region demonstrated an overall left-right asymmetry, both sides were combined and the area within each tracing was calculated. In all regions, the sections were carefully matched between animals. Cell counts and area measurements were averaged over the number of sections analyzed for each brain area, and cell density (number of cells per mm^2) was calculated for each subject. Group differences in the size of the region and the density of $BrdU^+$ cells for each region were analyzed either by a *t* test (experiment 1) or by a one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls (SNK) post-hoc test (experiments 2 and 3). Because the BrdU-labeled nuclei were $\approx$5 μm in diameter and sections at 120 μm intervals were counted, the possibility of counting split cells on different sections was minimized to less than 10%, according to the equation of Abercrombie (Guillery and Herrup, 1997).

BrdU-, TuJ1-, and NG2-labeled cells were quantified in the amygdala for experiments 1 and 2. Cells were visualized under 63X magnification using a Zeiss 510NLO confocal microscope. At least 170 cells were counted per group from two sections/animal. Individual cells stained for BrdU/TuJ1, BrdU/NG2, or BrdU alone were counted. Percentages were calculated for the individual subject's number of double-labeled cells divided by the corresponding subject's total number of $BrdU^+$ cells and multiplied by 100. Group differences in the percentage of $BrdU^+$ cells containing a neuronal (TuJ1) or glial (NG2) marker were analyzed by a one-way ANOVA.

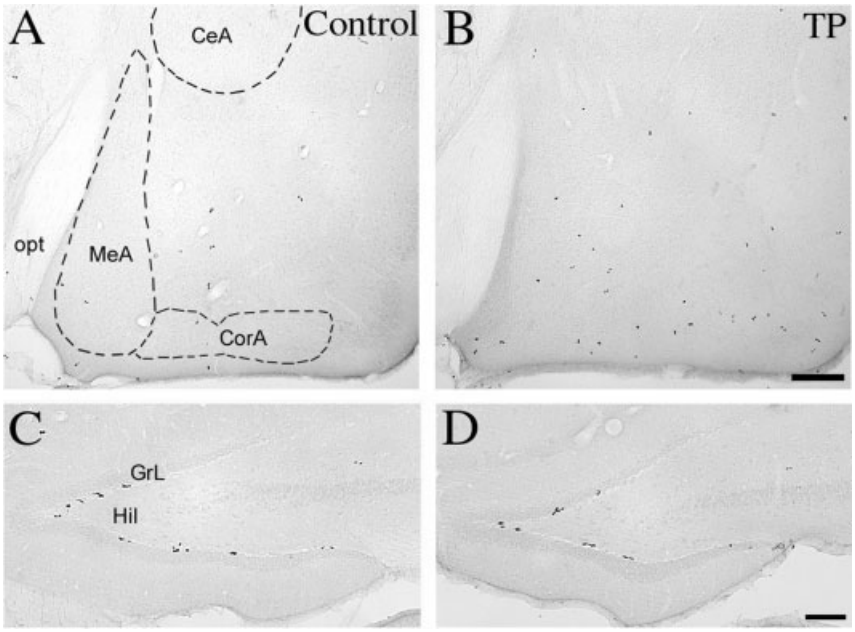

Figure 1 Photomicrographs of $BrdU^+$ cells following oil (control) or testosterone propionate (TP) treatment. (A,B) In the amygdala, the control group (A) shows less $BrdU^+$ cells than the TP-treated group (B). Scale bar = 200 μm. CeA, central nucleus; CorA, cortical nucleus; MeA, medial nucleus; opt, optic tract. (C,D) In the DG, the control group (C) did not differ from the TP-treated group (D). Scale bar = 100 μm. GrL, granule cell layer; Hil, hilus.

Radioimmunoassays (RIAs)

Prior to sacrifice, blood samples were obtained from the jugular vein. Blood was drawn with 20 μL of 0.2 *M* EDTA in the syringe to prevent clotting. Following collection, blood was placed in centrifuge tubes on ice and was then centrifuged at 3,000 rpm for 25 min. The resulting plasma was stored at −80°C. Plasma concentrations of testosterone were measured in duplicate by a Coat-a-Count total testosterone kit and of estradiol in duplicate by a Coat-a-Count estradiol kit (Diagnostic Products Corp.). For the testosterone kit, plasma from castrated male voles was added to the standards to control for nonspecific competition in male vole plasma and to prevent nonspecific binding of iodinated testosterone. Differing volumes of vole plasma were parallel to the standard curve of testosterone and estradiol, thus verifying the kits for use in voles. For the testosterone and estradiol assays, the sensitivities were 4 and 8 pg/mL, respectively, and the intra- and interassay coefficients of variation were both <5%. Sample values were analyzed by a *t* test or ANOVA, followed by a SNK post-hoc test.

RESULTS

Experiment 1

The size of each measured region did not differ between the two treatment groups ($p > 0.05$). BrdU immunocytochemistry produced dense nuclear staining of cells in specific regions of the vole brain. In the amygdala, the TP-treated males had a significantly higher density of $BrdU^+$ cells compared to the oil-treated group ($t = 3.81$; $p < 0.05$) [Figs. 1(A,B) and 2]. Specifically, the TP-treated group displayed a higher density of $BrdU^+$ cells in the cortical ($t = 3.13$; $p < 0.05$) and medial ($t = 5.47$; $p < 0.001$) nuclei, but not in the central nucleus, of the amygdala ($t = 0.74$; $p > 0.05$) [Fig. 2(B)]. Group differences were not found in the DG ($t = 0.24$; $p > 0.05$) and VMH ($t = 0.76$; $p > 0.05$) [Figs. 1(C,D) and 2(A)]. For the control group, the density of $BrdU^+$ cells in the DG was approximately 5.6-times the cell density in the amygdala and 6.8-times the cell density in the VMH. Following the TP-treatment, the density of $BrdU^+$ cells in the DG was approximately 2.4-times the cell density in the amygdala.

Experiment 2

In the amygdala, the EB-treated group had a significantly higher density of $BrdU^+$ cells than did the DHT- and oil-treated groups ($F = 7.52$; $p < 0.05$) [Fig. 3(A)]. Upon performing a post-hoc contrast test, the TP-treated group appeared to approach significance in having a higher density of $BrdU^+$ cells than

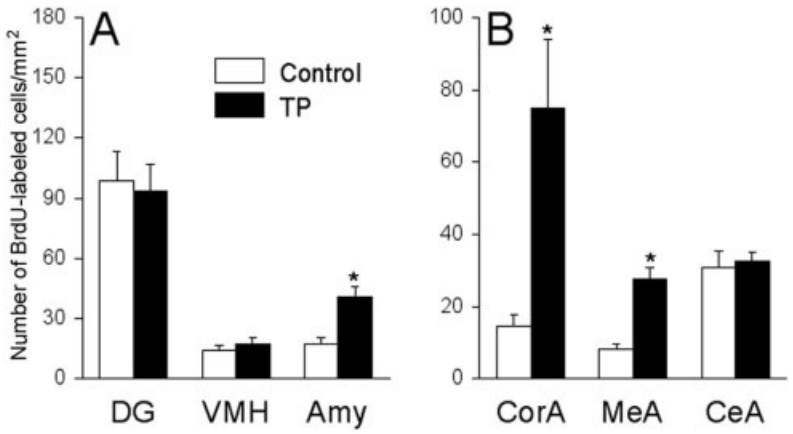

Figure 2 The effects of testosterone propionate (TP) treatment on the density of $BrdU^+$ cells for each area in male meadow voles. (A) TP treatment significantly increased the density of $BrdU^+$ cells in the amygdala (Amy), but not in the dentate gyrus of the hippocampus (DG) or ventromedial nucleus of the hypothalamus (VMH). (B) In the subnuclei of the amygdala, TP-treatment induced an increase in the density of $BrdU^+$ cells in the cortical (CorA) and medial (MeA), but not central (CeA), nuclei. $*p < 0.05$; error bars indicate standard error of the mean.

the oil-treated group ($p = 0.059$). For the specific subnuclei, the TP- and EB-treated groups had a higher density of $BrdU^+$ cells in the cortical ($F = 18.14$; $p < 0.001$) and medial ($F = 9.68$; $p < 0.001$) nuclei, but not in the central nucleus ($F = 3.07$; $p > 0.05$), of the amygdala than did the DHT- or oil-treated groups; the latter two groups did not differ from each other [Fig. 3(B)]. Group differences in the density of $BrdU^+$ cells were not seen in the DG ($F = 1.93$; $p > 0.05$) or VMH ($F = 0.63$; $p > 0.05$) [Fig. 3(A)]. Further, no group differences were found for the area of each brain region measured ($p > 0.05$).

The testosterone and estradiol RIAs confirmed group assignment. The TP-treated group had a higher level of plasma testosterone (11.4 ± 1.4 ng/mL) than did the oil- (0.8 ± 0.1 ng/mL) or DHT-treated groups

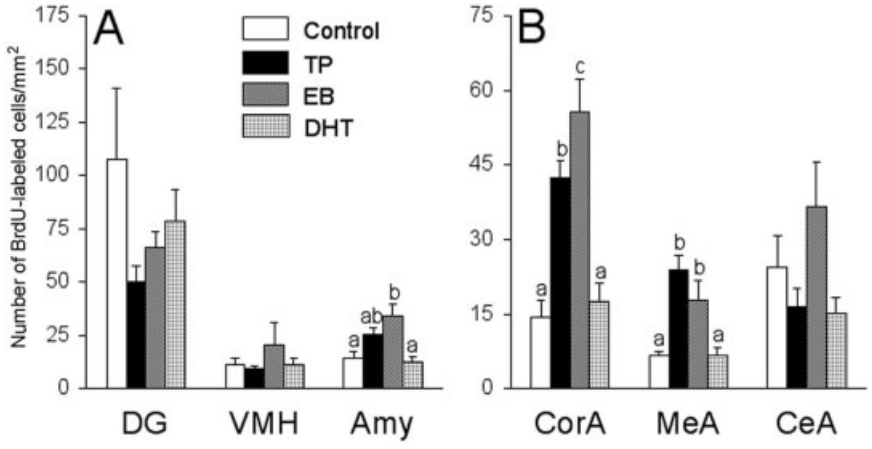

Figure 3 The effects of testosterone propionate (TP), estrogen benzoate (EB), or 5α-dihydrotestosterone (DHT) on the density of $BrdU^+$ cells for each area in male meadow voles. (A) EB treatment induced an increase in the density of $BrdU^+$ cells in the amygdala (Amy), but not the dentate gyrus of the hippocampus (DG) or ventromedial hypothalamus (VMH), as compared to the control. (B) Within subnuclei of the amygdala, an increase in the density of $BrdU^+$ cells following TP or EB treatment was present in the cortical (CorA) and medial (MeA), but not central (CeA), nuclei. Letters represent the results of the post-hoc test; nonshared letters indicate statistical difference at $p < 0.05$; and error bars indicate standard error of the mean.

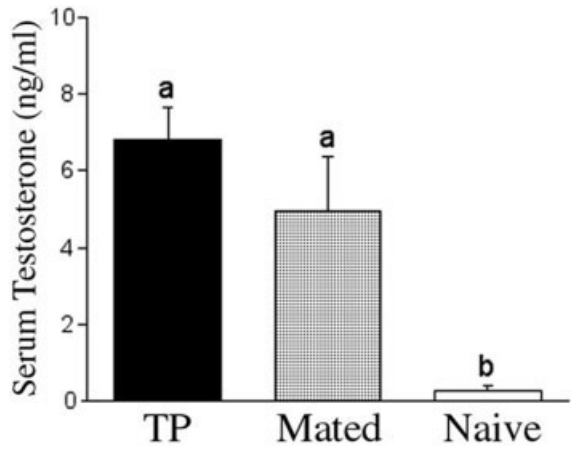

Figure 4 Mean plasma testosterone levels in meadow voles. Testosterone propionate (TP)-treated males displayed levels similar to males that had mated with a female (Mated), both of which were significantly higher than sexually naive males (Naive). Letters represent the results of the post-hoc test; nonshared letters indicate statistical difference at $p < 0.05$; and error bars indicate standard error of the mean.

(0.9 ± 0.2 ng/mL) ($F = 74.56$; $p < 0.001$). An additional testosterone RIA was then performed to compare TP-treated values against naturally occurring testosterone levels. The plasma testosterone levels of TP-treated males ($n = 5$) did not differ from males sacrificed after mating ($n = 3$), and both were higher than sexually naive males ($n = 3$) ($F = 12.06$; $p < 0.05$) (Fig. 4). The EB-treated group had a higher level of plasma estradiol (1.0 ± 0.3 ng/mL) than did the oil-treated group (less than detection limits of 8 pg/mL).

To confirm phenotypes, new cells were labeled with BrdU and a neuronal (TuJ1) or glial (NG2) marker (Fig. 5). No differences were found in the percentage of $BrdU^+$ cells colocalized with either TuJ1 or NG2. Overall, approximately 44.2% of the cells labeled for BrdU and TuJ1, 34.5% for BrdU and NG2, and 21.3% for BrdU alone (Table 1). The 21.3% of BrdU-labeled cells that did not colabel with TuJ1 or NG2 may indicate undifferentiated progenitors, differentiated cells having not yet begun to express either of these markers, mature neurons that may not express TuJ1, or mature glial cells no longer containing NG2. Thus, the actual percentage of new neurons or glia may be higher depending on the eventual fate of these cells or the presence of other markers.

Experiment 3

A time course experiment was performed on intact, untreated males to investigate whether cells proliferate locally within the amygdala or VMH. On coronal and sagittal sections, $BrdU^+$ cells were visualized in the amygdala and VMH at 30 min, 1, 6, and 24 h following an acute injection of BrdU [Fig. 7(A)]. $BrdU^+$ cells were then quantified on the coronal sections (Fig. 6). In the amygdala, the 1 and 6 h groups did not differ from each other; however, both groups had a density of $BrdU^+$ cells that was higher than the 30 min group but lower than the 24 h group ($F = 13.25$; $p < 0.05$). In the MeA, the 24 h group had a higher density of cells than did the 30 min group ($F = 4.10$; $p < 0.05$). Group differences in BrdU cell density were not found in the CorA ($F = 0.15$; $p > 0.05$) or CeA ($F = 2.74$; $p > 0.05$) or in the VMH ($F = 1.31$; $p > 0.05$). Prior reports have described a "stream" of cells migrating into the cerebral cortex (Gould et al., 1999b); however, upon qualitative analysis, we could not visually identify any observable migratory stream pattern from the SVZ into the amygdala at these time points. BrdU-labeling was considered specific because labeled cells could not be found in animals that did not receive BrdU injections (negative control). Further, BrdU/TuJ1/NG2 immunolabeling was also performed to identify the phenotype of the cells present 30 min following the BrdU injection. Cells colocalized with BrdU/TuJ1 and BrdU/NG2 were identified within the amygdala at this time point [Fig. 7(B)].

DISCUSSION

In the present study, we found that TP or EB, but not DHT, exerted site-specific increases in the density of new cells in the amygdala of adult male meadow voles. Plasma testosterone levels in the TP-treated males were similar to males following mating, indicating that hormonal treatment reached physiological levels. The majority of the new cells displayed neuronal (≈42%) or glial (≈35%) phenotypes. Finally, new cells were identified in the amygdala and VMH as early as 30 min following an acute injection of

Table 1 Percentage ± SEM of $BrdU^+$ Cells Double-Labeled with a Neuronal (TuJ1) or Glial (NG2) Marker in the Amygdala

Treatment	BrdU/TuJ1	BrdU/NG2	BrdU Alone
Control	41.0 ± 9.4	36.8 ± 12.3	22.2 ± 5.1
TP	32.8 ± 8.9	46.4 ± 6.4	20.9 ± 6.2
EB	44.7 ± 7.1	33.7 ± 6.5	21.6 ± 3.0
DHT	58.3 ± 15.9	21.0 ± 7.9	20.8 ± 9.1
Overall	44.2%	34.5%	21.3%

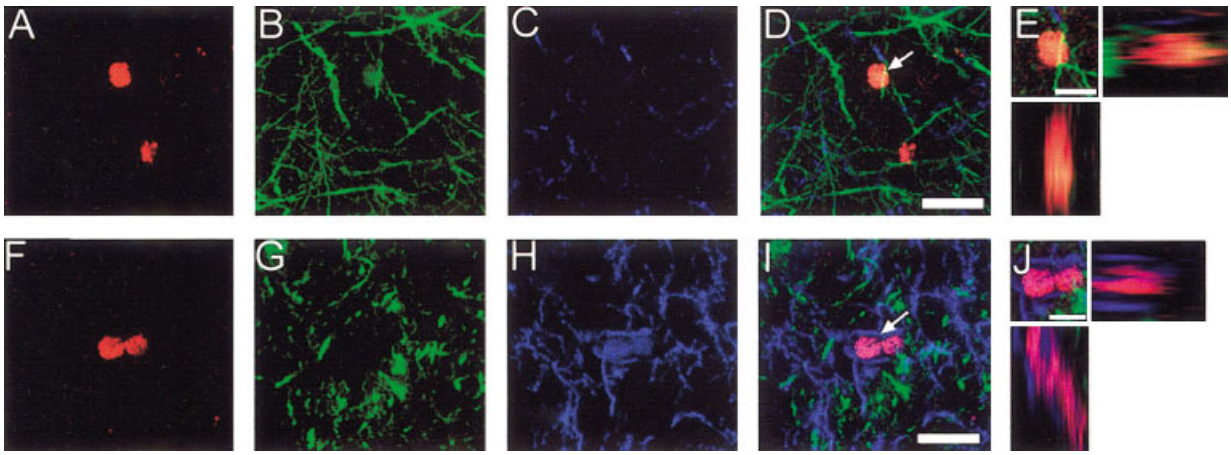

Figure 5 Confocal laser microscope images of cells stained for BrdU, TuJ1, and/or NG2 in the amygdala of male meadow voles. (A–D) Images display staining for (A) BrdU (red), (B) TuJ1 (green), (C) NG2 (blue), and (D) all three markers. BrdU and TuJ1 colocalized cells display a yellow image (arrow), and a BrdU-only labeled cell displays a red image. Scale bar = 10 μm. (E) Zoomed image of newborn neuron. Views along the *y*-*z* axis (right) and *x*-*z* axis (below) demonstrate 3D colocalization of BrdU and TuJ1. Scale bar = 5 μm. (F–I) Images display staining for (F) BrdU (red), (G) TuJ1 (green), (H) NG2 (blue), and (I) all three markers. BrdU and NG2 colocalized cells display a purple image (arrow). Scale bar = 10 μm. (J) Zoomed image of newborn glial cells. Views along the *y*-*z* axis (right) and *x*-*z* axis (below) demonstrate 3D colocalization of BrdU and NG2. Scale bar = 5 μm.

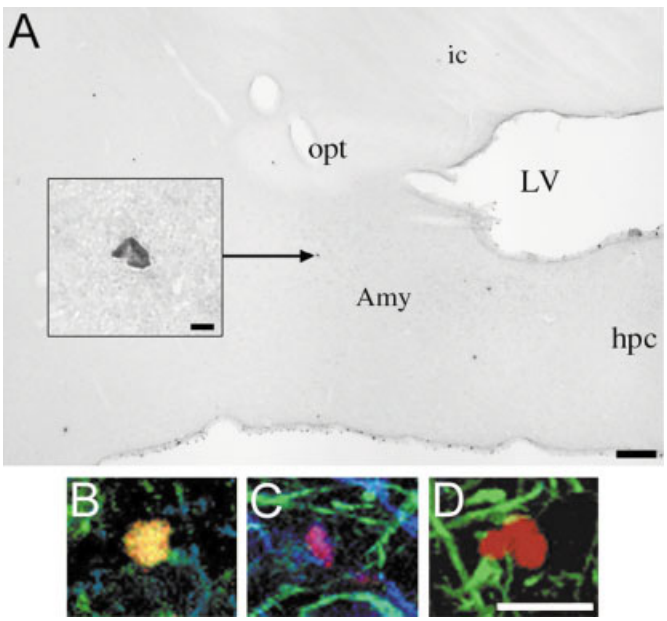

Figure 7 (A) Sagittal section photomicrograph of $BrdU^+$ cells in the amygdala (Amy) 30 min after an acute injection of BrdU. Scale bar = 100 μm. The insert displays a higher magnification of BrdU-labeling within the amygdala. Scale bar = 5 μm. hpc, hippocampal complex; ic, internal capsule; LV, lateral ventricle; opt, optic tract. (B–D) Confocal laser microscope images of cells stained for BrdU, TuJ1, and/or NG2 in the amygdala 30 min after an acute injection of BrdU. Cells displayed staining for (B) BrdU/TuJ1, (C) BrdU/NG2, and (D) BrdU alone. Scale bar = 10 μm.

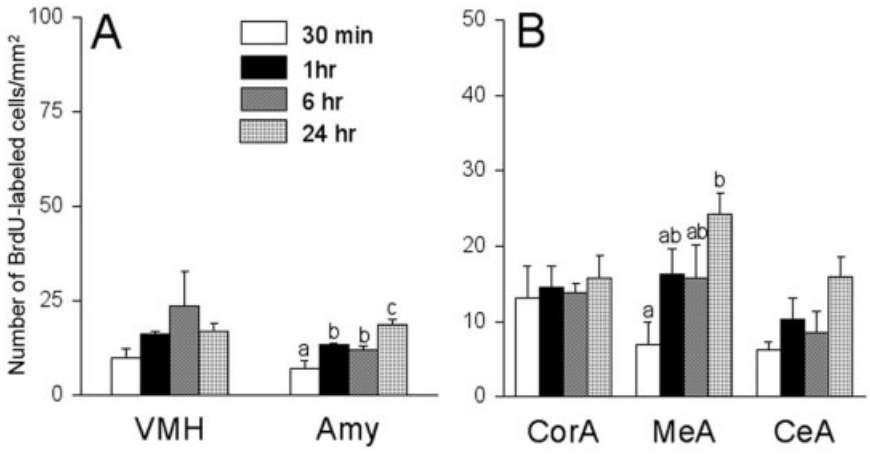

Figure 6 The density of $BrdU^+$ cells at 30 min, 1, 6, and 24 h following an acute BrdU injection. (A) In the amygdala (Amy), the 1 and 6 h groups did not differ from each other; however, both groups had a density of $BrdU^+$ cells that was higher than the 30 min group but lower than the 24 h group. Significant differences were not found in the ventromedial hypothalamus (VMH). (B) Within subnuclei of the amygdala, in the medial nucleus (MeA), the 24 h group had a higher density of $BrdU^+$ cells than did the 30 min group, but the 1 and 6 h groups did not differ from any other groups. Differences were not found in the cortical (CorA) or central (CeA) nuclei. Letters represent the results of the post-hoc test; nonshared letters indicate statistical difference at $p < 0.05$; and error bars indicate standard error of the mean.

BrdU. Together, these data suggest that gonadal steroid hormones affect the number of newly proliferated cells in the amygdala, most likely by acting through an estrogenic mechanism, and these effects may be exerted on cells locally proliferating within the amygdala.

Gonadal Steroid Hormones Influence Cell Proliferation

In the mammalian brain, testosterone may exert its effects directly or via aromatization into estrogen (Naftolin et al., 1975) or reduction into DHT (Lieberburg and McEwen, 1977). We found that TP or EB treatment increased the density of new cells in the amygdala, particularly in the MeA and CorA. In contrast, DHT treatment was ineffective. The similarities between the TP and EB, but not DHT, effects indicate that testosterone may act through an estrogen receptor mechanism to affect the proliferation of new cells in the amygdala. It is interesting to note that testosterone and its metabolites also act on certain reproductive behaviors and neurotransmitters in a similar manner. For example, in male hamsters, implants of testosterone or estradiol, but not DHT, in the MeA stimulate sexual activity in castrated males (Wood and Newman, 1995; Wood, 1996), and aromatase inhibition decreases mating-associated behaviors (Steel and Hutchison, 1987). In gonadectomized rats, vasopressin mRNA in the MeA and serotonin receptor and transporter mRNAs in the dorsal raphe nucleus display an increased expression with testosterone or estrogen, but not DHT, treatment (Wang and De Vries, 1995; Fink et al., 1999). However, if DHT is given in combination with estrogen, it increases the number of AVP mRNA labeled cells in MeA (Wang and De Vries, 1995). Because androgen receptor immunoreactivity in the MeA of castrated male rats can be increased by estrogen treatment (Lynch and Story, 2000), the effects of DHT on the central nervous system may rely on the presence of estrogen (DeVries et al., 1994). Therefore, we cannot exclude the possibility that DHT may be able to act synergistically with estrogen to influence cell proliferation.

Several lines of evidence support this possibility of local gonadal steroid action within the amygdala. First, estrogen-receptor containing cells are present in the amygdala, particularly in the MeA and CorA, in a variety of rodent species, including rats (Pfaff and Keiner, 1973; Simerly et al., 1990), hamsters (Wood et al., 1992), and prairie voles (Hnatczuk et al., 1994). Second, high levels of aromatase activity are found in the MeA and CorA of male rats, and castration does not affect these levels (Roselli et al., 1984; Roselli and Resko, 1987). After testosterone infusions in castrated male rats, high levels of estrogen can be found in the amygdala (Lieberburg and McEwen, 1977). Finally, testosterone or estrogen treatment has been found to

affect the neuroanatomy of the amygdala in rodents, possibly due to changes in neuronal soma size or neurogenesis. In female rats, estrogen treatment prior to postnatal day 30 increases MeA volume (Mizukami et al., 1983), and in adult, castrated male hamsters, testosterone administration also increases MeA volume (Cooke et al., 2002). Interestingly, the latter finding could not be attributed to changes in soma size, presenting the possibility of increased neurogenesis in response to testosterone for this species.

The underlying mechanisms for the steroid regulation of cell proliferation in adulthood are still unknown. However, because steroid hormones are able to alter the transcription rates of genes (Simerly, 1990), one may speculate that estrogen can induce or facilitate progenitor cells to enter the cell cycle. Alternatively, estrogen may act on other cells, which, in turn, cause local progenitor cells to proliferate. For instance, astrocytes contain steroid hormone receptors (Finley and Kritzer, 1999) and have been shown to induce cell proliferation *in vitro* (Lim and Alvarez-Buylla, 1999; Song et al., 2002). In response to steroid hormones, astrocytes may secrete neurochemicals such as BDNF (Ikeda et al., 2001), which has been shown to enhance cell proliferation and survival in the adult brain (Pencea et al., 2001). Importantly, in prairie voles, a species closely related to meadow voles, increased BDNF expression is present in the amygdala following estrogen treatment (Liu et al., 2001). Thus, if stem or progenitor cells are locally situated within the amygdala, it is feasible that local astrocytes function to induce these cells to undergo proliferation.

The TP-treated and mated males had similar levels of plasma testosterone, both of which were higher than sexually naive males in the present study or males captured during the breeding season in a previous experiment ($\approx$1.8 ng/mL; Galea and McEwen, 1999). These data suggest that mating was accompanied by an increase in plasma testosterone, and the TP treatment resulted in a level of testosterone similar to naturally occurring physiological levels following mating in male meadow voles. Indeed, reports have indicated that testosterone levels become elevated significantly after ejaculation in the closely related male prairie voles (increase from $\approx$1.4 to 8.8 ng/mL; Gaines et al., 1985), and a comparable level of testosterone in male meadow voles increases the attractiveness of male odors to females (Ferkin et al., 1994). A drawback in the present study was that blood samples were only taken at sacrifice, so it was unclear whether this level of testosterone was consistent throughout the 72 h of treatment. Therefore, the observed effects on cell proliferation may be due to the initial rising levels of infused TP, maintained infusion rate, or maximal level of TP released.

New Cells Proliferate Locally within the Amygdala

In a previous study of rats, the rate of migration for new cells derived from the SVZa into the olfactory bulb was calculated as 22.8 μm/h (Luskin and Boone, 1994). If we assume the same rate for migration into the amygdala, we would expect it to take about 13 h for the cell in Figure 7(A) to migrate from the nearest lateral ventricle into the amygdala. Certainly, we cannot exclude the possibility that new cells from the SVZa could migrate into the amygdala much faster, but this rate would need to be at least 26 times faster than the rate into the olfactory bulb. Therefore, because $BrdU^+$ cells were identified in the amygdala 30 min postinjection, BrdU may only be incorporated during S-phase of the cell cycle, and a very high rate of migration would be necessary for migration from a ventricle, one may infer with high probability that these cells are dividing locally, rather than dividing and then migrating from a ventricular region. Furthermore, these data suggest that the differences seen in experiments 1 and 2 occurred due to the actions of the hormones on locally proliferating progenitors. However, we are currently unable to draw a definitive conclusion about the presence of stem cells within the amygdala. It has been estimated that stem cells proliferate approximately once every 4 weeks (Morshead et al., 1994), producing progenitor cells that may continue to progress through subsequent divisions $\approx$17 h while migrating (Smith and Luskin, 1998). Thus, it is possible that stem cells are located in the better-known proliferative population, such as the SVZ, and produce progenitors that migrate to the amygdala and continue to proliferate locally within this region. On the other hand, we cannot discount the possibility that stem cells are localized within the adult amygdala, a notion that is currently debatable. Some data suggest that stem cells are not localized outside of the ventricular subependyma, for example, within the adult DG (Seaberg and van Der Kooy, 2002), whereas others suggest that stem cells are present in the DG and in other non-neurogenic zones of the adult brain (Palmer et al., 1997, 1999).

Possible Functional Significance of New Cells in the Amygdala

The amygdala has been implicated in many reproductive-associated functions and behaviors, such as olfactory/pheromonal processing (Luiten et al., 1985;

Meredith, 1991), social learning and memory (Kirkpatrick et al., 1994; Demas et al., 1997), copulatory actions (Harris and Sachs, 1975; Dominguez et al., 2001), and aggressive behavior (Albert and Walsh, 1984; Wang et al., 1997). In the present study, TP or EB treatment increased the density of new cells in the amygdala of adult male meadow voles; importantly, the TP treatment induced plasma testosterone levels similar to levels found following mating, indicating the physiological relevance of the findings. In male meadow voles, testosterone plays an important role in a male's attractiveness to a female (Ferkin et al., 1994) and preference for a specific female's odor (Ferkin, 1992). Periods of reproduction have been correlated with high levels of testosterone and aggression (Boonstra et al., 1994; Galea and McEwen, 1999), and aggression increases a male's reproductive success (Storey and Snow, 1990). Although not known in meadow voles, direct implants of testosterone into the MeA enhance sexual activity in male hamsters (Wood and Newman, 1995), and the amygdala is implicated in aggressive and social behaviors in male prairie voles (Kirkpatrick et al., 1994; Wang et al., 1997). Therefore, it is possible that new cells in the amygdala may mature and contribute to one or more of these reproductive-associated functions and/or behaviors. Recent findings appear to support the concept that adult-born neurons mature and function in the brain: in male golden hamsters, new neurons in the olfactory bulb become activated following exposure to females in estrus (Huang and Bittman, 2002), and in mice, a diminished number of new cells in the DG is correlated with deficits in learning and memory abilities (Shors et al., 2001).

CONCLUSION

In male meadow voles, hormonal status influences the density of new neurons and glia in the MeA and CorA; this effect was site-specific, as differences were not seen in the DG and VMH. Because increases were seen with TP and EB, but not DHT, and because progenitors appear to be proliferating within the amygdala, it is hypothesized that the hormonal effects on new cells are most likely occurring via estrogenic mechanisms locally within the amygdala. In mammals, including humans, gonadal hormones are involved in the development, differentiation, and protection of the central nervous system (Garcia-Segura et al., 1994; Romeo et al., 2002), and the amygdala has been implicated in a variety of cognitive and behavioral functions (Albert and Walsh, 1984; Cahill et al., 1996). Therefore, in future studies, it will be both interesting and essential to determine the functional significance of new cell incorporation in the adult amygdala.

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