

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

Christie D. Fowler,¹ Marc E. Freeman,² Zuoxin Wang¹

¹ Department of Psychology, Program in Neuroscience, Florida State University, Tallahassee, Florida 32306

² Department of Biological Sciences, Program in Neuroscience, Florida State University, Tallahassee, Florida 32306

Received 9 December 2002; accepted 19 May 2003

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 5 α -dihydrotestosterone (DHT) was ineffective. A larger proportion ($\approx 44\%$) of the BrdU-labeled cells in the amygdala displayed a neuronal phenotype, and a

lesser percentage ($\approx 35\%$) 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

Keywords: neurogenesis; testosterone; estrogen; DHT; meadow vole

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).

Correspondence to: C. D. Fowler (fowler@psy.fsu.edu).

Contract grant sponsor: NIH; contract grant numbers: MH-64352 and NS-07437 (C.D.F.), MH-58616 and MH-66734 (Z.X.W.), and DK-43200 (M.E.F.).

© 2003 Wiley Periodicals, Inc.

DOI 10.1002/neu.10273

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 5 α -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 × 18 × 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 ± 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/ μ L sesame oil, $\approx 20 \mu$ L 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/ μ L oil), DHT ($n = 7$; 0.1 mg/ μ L oil), or estradiol benzoate (EB; $n = 6$; ≈ 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 μ g/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 μ g/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 μ m 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 were 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 X-100 in 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 $\approx 5 \mu\text{m}$ 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.

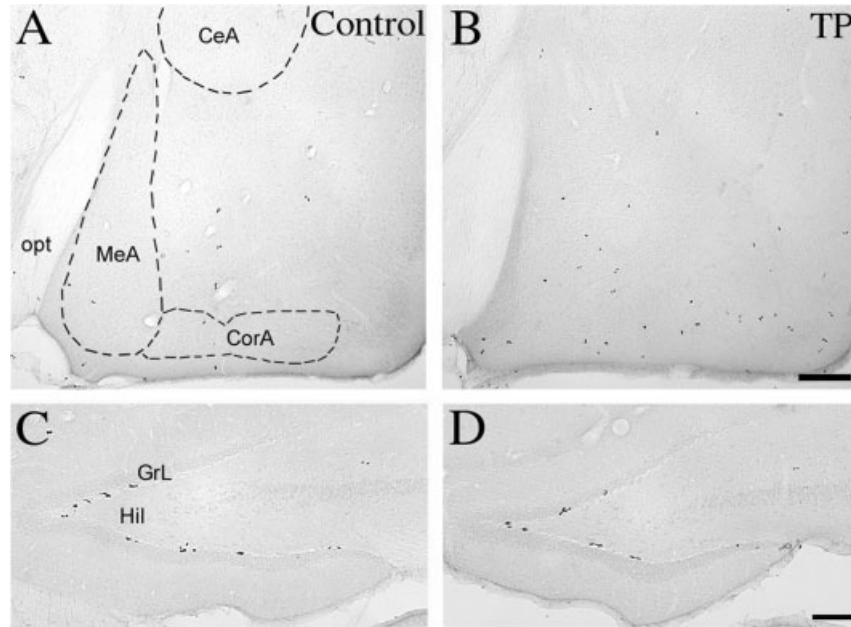


Figure 1 Photomicrographs of BrdU⁺ cells following oil (control) or testosterone propionate (TP) treatment. (A,B) In the amygdala, the control group (A) shows less BrdU⁺ cells than the TP-treated group (B). Scale bar = 200 μ m. CeA, central nucleus; CorA, cortical nucleus; MeA, medial nucleus; opt, optic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

Radioimmunoassays (RIAs)

Prior to sacrifice, blood samples were obtained from the jugular vein. Blood was drawn with 20 μ L 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

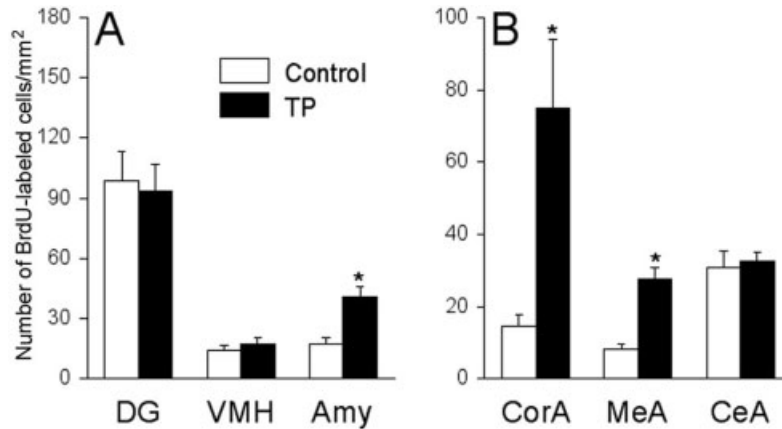


Figure 2 The effects of testosterone propionate (TP) treatment on the density of BrdU⁺ cells for each area in male meadow voles. (A) TP treatment significantly increased the density of BrdU⁺ cells in the amygdala (Amy), but not in the dentate gyrus of the hippocampus (DG) or ventromedial nucleus of the 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

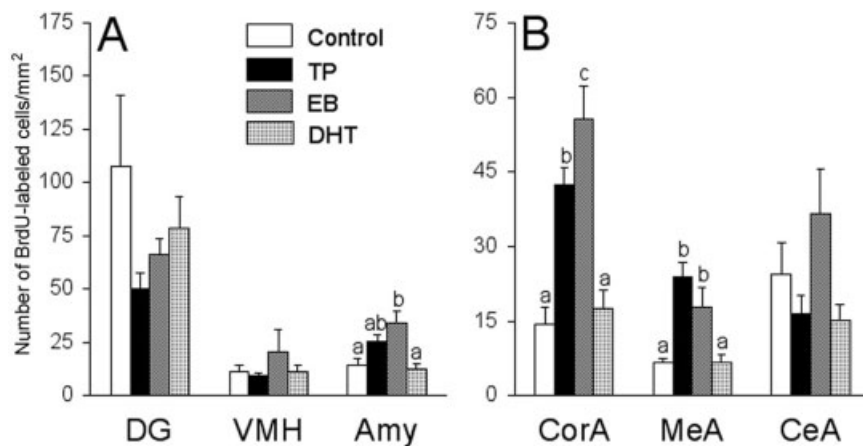


Figure 3 The effects of testosterone propionate (TP), estrogen benzoate (EB), or 5α -dihydrotestosterone (DHT) on the density of BrdU⁺ cells for each area in male meadow voles. (A) EB treatment induced an increase in the density of BrdU⁺ cells in the amygdala (Amy), but not the dentate gyrus of the hippocampus (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.

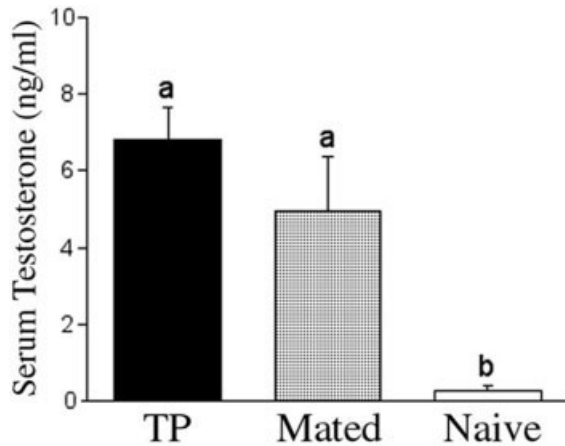


Figure 4 Mean plasma testosterone levels in meadow voles. Testosterone propionate (TP)-treated males displayed levels similar to males that had mated with a female (Mated), both of which were significantly higher than sexually naive males (Naive). Letters represent the results of the post-hoc test; nonshared 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 prolifer-

ate 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 ($\approx 42\%$) or glial ($\approx 35\%$) phenotypes. Finally, new cells were identified in the amygdala and VMH as early as 30 min following an acute injection of

Table 1 Percentage \pm 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 \pm 9.4	36.8 \pm 12.3	22.2 \pm 5.1
TP	32.8 \pm 8.9	46.4 \pm 6.4	20.9 \pm 6.2
EB	44.7 \pm 7.1	33.7 \pm 6.5	21.6 \pm 3.0
DHT	58.3 \pm 15.9	21.0 \pm 7.9	20.8 \pm 9.1
Overall	44.2%	34.5%	21.3%

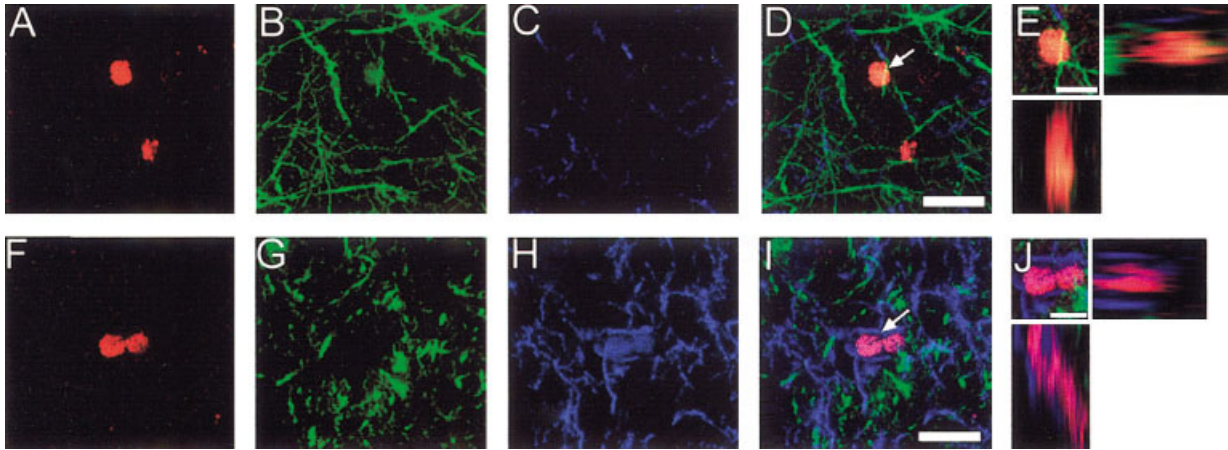


Figure 5 Confocal laser microscope images of cells stained for BrdU, TuJ1, and/or NG2 in the amygdala of male meadow voles. (A–D) Images display staining for (A) BrdU (red), (B) TuJ1 (green), (C) NG2 (blue), and (D) all three markers. BrdU and TuJ1 colocalized cells display a yellow image (arrow), and 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 .

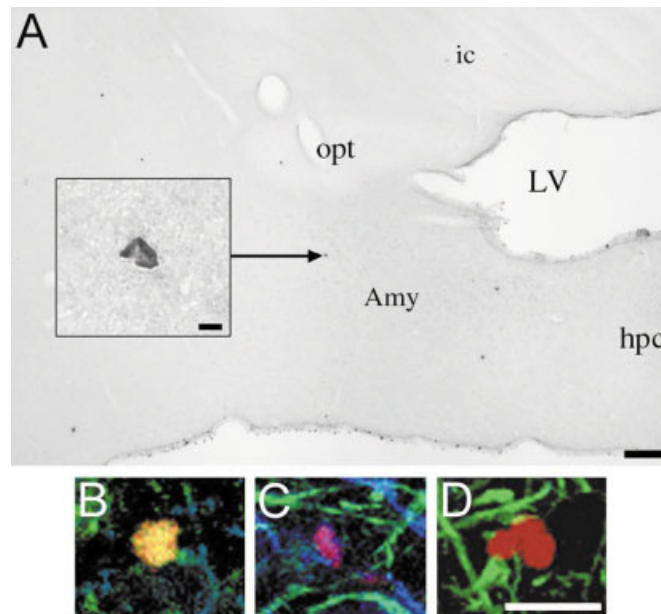


Figure 7 (A) Sagittal section photomicrograph of BrdU⁺ cells in the amygdala (Amy) 30 min after an acute injection of BrdU. Scale bar = 100 μm . The insert displays a higher magnification of BrdU-labeling within the amygdala. Scale bar = 5 μm . hpc, hippocampal complex; ic, internal capsule; LV, lateral 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 .

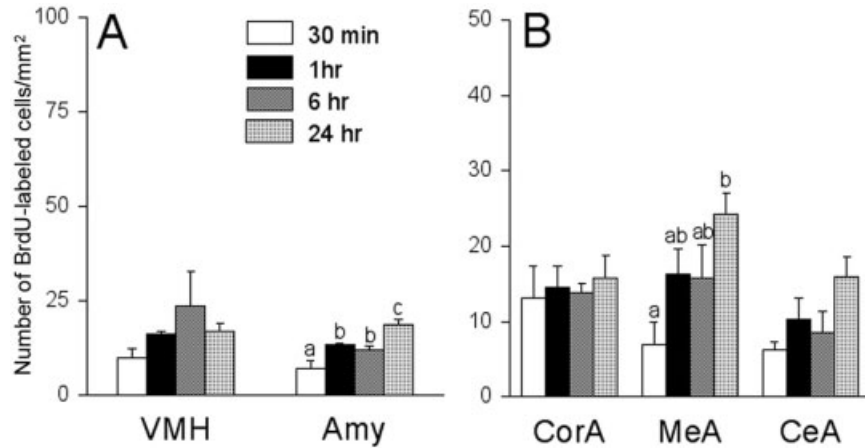


Figure 6 The density of BrdU⁺ cells at 30 min, 1, 6, and 24 h following an acute BrdU injection. (A) In the amygdala (Amy), the 1 and 6 h groups did not differ from each other; however, both groups had a density of BrdU⁺ cells that was higher than the 30 min group but lower than the 24 h group. Significant 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, vasopres-

sin 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 (Hnatzuk 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 (≈ 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 ≈ 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 $\mu\text{m}/\text{h}$ (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 ≈ 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 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.

We are grateful to Dr. Yan Liu, Cheryl Pye, and Jenny Stowe for their technical assistance and to Dr. Thomas Curtis, Dr. Yan Liu, Brandon Aragona, and Jenny Stowe for a critical reading of the manuscript. We also thank Dr. Theresa Lee of the University of Michigan for providing the meadow vole colony.

REFERENCES

- Albert DJ, Walsh ML. 1984. Neural systems and the inhibitory modulation of agonistic behavior: a comparison of mammalian species. *Neurosci Biobehav Rev* 8:5–24.
- Alexander JE, Hunt DF, Lee MK, Shabanowitz J, Michel H, Berlin SC, MacDonald TL, Sundberg RJ, Rebhun LI, Frankfurter A. 1991. Characterization of posttranslational modifications in neuron-specific class III beta-tubulin by mass spectrometry. *Proc Natl Acad Sci USA* 88:4685–4689.
- Bedard A, Bernier PJ, Vinet J, Levesque M, Goulet S, Parent A. 2001. Neuroblast migratory stream in the temporal lobe of the adult primate. In: Program No. 248.8, Abstracts Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience. CD-ROM.
- Boonstra R, Hochachka WM, Pavone L. 1994. Heterozygosity, aggression, and population fluctuations in meadow voles (*Micortus pennsylvanicus*). *Evolution* 48:1350–1363.
- Brezun JM, Daszuta A. 1999. Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience* 89:999–1002.
- Brezun JM, Daszuta A. 2000. Serotonin may stimulate granule cell proliferation in the adult hippocampus, as observed in rats grafted with foetal raphe neurons. *Eur J Neurosci* 12:391–396.
- Butt AM, Berry M. 2000. Oligodendrocytes and the control of myelination in vivo: new insights from the rat anterior medullary velum. *J Neurosci Res* 59:477–488.
- Cahill L, Haier RJ, Fallon J, Alkire MT, Tang C, Keator D, Wu J, McGaugh JL. 1996. Amygdala activity at encoding correlated with long-term, free recall of emotional information. *Proc Natl Acad Sci USA* 93:8016–8021.
- Cameron HA, Gould E. 1994. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* 61:203–209.
- Cameron HA, McKay RD. 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol* 435:406–417.
- Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. 2000. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci* 20:6404–6412.

- Cooke BM, Hegstrom CD, Breedlove SM. 2002. Photoperiod-dependent response to androgen in the medial amygdala of the Siberian hamster, *Phodopus sungorus*. *J Biol Rhythms* 17:147–154.
- Coolen LM, Wood RI. 1999. Testosterone stimulation of the medial preoptic area and medial amygdala in the control of male hamster sexual behavior: redundancy without amplification. *Behav Brain Res* 98:143–153.
- Dawson MR, Levine JM, Reynolds R. 2000. NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? *J Neurosci Res* 61:471–479.
- Demas GE, Williams JM, Nelson RJ. 1997. Amygdala but not hippocampal lesions impair olfactory memory for mate in prairie voles (*Microtus ochrogaster*). *Am J Physiol* 273:R1683–1689.
- DeVries GJ, Wang Z, Bullock NA, Numan S. 1994. Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats. *J Neurosci* 14:1789–1794.
- Dominguez J, Riolo JV, Xu Z, Hull EM. 2001. Regulation by the medial amygdala of copulation and medial preoptic dopamine release. *J Neurosci* 21:349–355.
- Ferkin MH. 1992. Time course of androgenic modulation of odor preferences and odor cues in male meadow voles, *Microtus pennsylvanicus*. *Horm Behav* 26:512–521.
- Ferkin MH, Sorokin ES, Renfro MW, Johnston RE. 1994. Attractiveness of male odors to females varies directly with plasma testosterone concentration in meadow voles. *Physiol Behav* 55:347–353.
- Fidler PS, Schuette K, Asher RA, Dobbertin A, Thornton SR, Calle-Patino Y, Muir E, Levine JM, Geller HM, Rogers JH, et al. 1999. Comparing astrocytic cell lines that are inhibitory or permissive for axon growth: the major axon-inhibitory proteoglycan is NG2. *J Neurosci* 19:8778–8788.
- Fink G, Sumner B, Rosie R, Wilson H, McQueen J. 1999. Androgen actions on central serotonin neurotransmission: relevance for mood, mental state and memory. *Behav Brain Res* 105:53–68.
- Finley SK, Kritzer MF. 1999. Immunoreactivity for intracellular androgen receptors in identified subpopulations of neurons, astrocytes and oligodendrocytes in primate prefrontal cortex. *J Neurobiol* 40:446–457.
- Fowler CD, Liu Y, Ouimet C, Wang Z. 2002. The effects of social environment on adult neurogenesis in the female prairie vole. *J Neurobiol* 51:115–128.
- Fuchs E, Flugge G, Ohl F, Lucassen P, Vollmann-Honsdorf GK, Michaelis T. 2001. Psychosocial stress, glucocorticoids, and structural alterations in the tree shrew hippocampus. *Physiol Behav* 73:285–291.
- Gaines MS, Fugate CL, Johnson ML, Johnson DC, Hisey JR, Quadagno D. 1985. Manipulation of aggressive behavior in male prairie voles (*Microtus ochrogaster*) implanted with testosterone in silastic tubing. *Can J Zool* 63:2525–2528.
- Galea LAM, McEwen BS. 1999. Sex and seasonal differences in the rate of cell proliferation in the dentate gyrus of adult wild meadow voles. *Neurosci* 89:955–964.
- Garcia-Segura LM, Chowen JA, Parducz A, Naftolin F. 1994. Gonadal hormones as promoters of structural synaptic plasticity: cellular mechanisms. *Prog Neurobiol* 44:279–307.
- Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. 1999a. Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci* 2:260–265.
- Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E. 1997. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* 17:2492–2498.
- Gould E, Reeves AJ, Graziano MS, Gross CG. 1999b. Neurogenesis in the neocortex of adult primates. *Science* 286:548–552.
- Guillery RW, Herrup K. 1997. Quantification without pontification: choosing a method for counting objects in sectioned tissues. *J Comp Neurol* 386:2–7.
- Harris VS, Sachs BD. 1975. Copulatory behavior in male rats following amygdaloid lesions. *Brain Res* 86:514–518.
- Hnatczuk OC, Lisciotto CA, DonCarlos LL, Carter CS, Morrell JI. 1994. Estrogen receptor immunoreactivity in specific brain areas of the prairie vole (*Microtus ochrogaster*) is altered by sexual receptivity and genetic sex. *J Neuroendocrinol* 6:89–100.
- Huang L, Bittman EL. 2002. Olfactory bulb cells generated in adult male golden hamsters are specifically activated by exposure to estrous females. *Horm Behav* 41:343–350.
- Huang L, DeVries GJ, Bittman EL. 1998. Photoperiod regulates neuronal bromodeoxyuridine labeling in the brain of a seasonally breeding mammal. *J Neurobiol* 36:410–420.
- Ikeda O, Murakami M, Ino H, Yamazaki M, Nemoto T, Koda M, Nakayama C, Moriya H. 2001. Acute up-regulation of brain-derived neurotrophic factor expression resulting from experimentally induced injury in the rat spinal cord. *Acta Neuropathol (Berl)* 102:239–245.
- Insel TR. 1990. Regional induction of c-fos-like protein in rat brain after estradiol administration. *Endocrinology* 126:1849–1853.
- Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J Neurosci* 22:2792–2803.
- Kameda Y, Kameya T, Frankfurter A. 1993. Immunohistochemical localization of a neuron-specific β tubulin isotype in the developing chicken ultimobranchial glands. *Brain Res* 628:121–127.
- Kempermann G, Kuhn HG, Gage FH. 1997. More hippocampal neurons in adult mice living in an enriched environment. *Nature* 386:493–495.
- Kirkpatrick B, Carter CS, Newman SW, Insel TR. 1994. Axon-sparing lesions of the medial nucleus of the amygdala decrease affiliative behaviors in the prairie vole

- (*Microtus ochrogaster*): behavioral and anatomical specificity. *Behav Neurosci* 108:501–513.
- Leranth C, Roth RH, Elsworth JD, Naftolin F, Horvath TL, Redmond Jr DE. 2000. Estrogen Is Essential for Maintaining Nigrostriatal Dopamine Neurons in Primates: Implications for Parkinson's Disease and Memory. *J Neurosci* 20:8604–8609.
- Lieberburg I, McEwen BS. 1977. Brain cell nuclear retention of testosterone metabolites, 5 α -dihydrotestosterone and estradiol-17 β , in adult rats. *Endocrinology* 100:588–597.
- Lim DA, Alvarez-Buylla A. 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *Proc Natl Acad Sci USA* 96:7526–7531.
- Liu Y, Fowler CD, Young LJ, Yan Q, Insel TR, Wang ZX. 2001. Expression and estrogen regulation of brain-derived neurotrophic factor gene and protein in the forebrain of female prairie voles. *J Comp Neurol* 433:499–514.
- Luiten PG, Koolhaas JM, de Boer S, Koopmans SJ. 1985. The cortico-medial amygdala in the central nervous system organization of agonistic behavior. *Brain Res* 332:283–297.
- Luskin MB, Boone MS. 1994. Rate and pattern of migration of lineally related olfactory bulb interneurons generated postnatally in the subventricular zone of the rat. *Chem Senses* 19:695–714.
- Lynch CS, Story AJ. 2000. Dihydrotestosterone and estrogen regulation of rat brain androgen-receptor immunoreactivity. *Physiol Behav* 69:445–453.
- Mallon BS, Shick HE, Kidd GJ, Macklin WB. 2002. Proteolipid promoter activity distinguishes two populations of NG2-positive cells throughout neonatal cortical development. *J Neurosci* 22:876–885.
- Meredith M. 1991. Sensory processing in the main and accessory olfactory systems: comparisons and contrasts. *J Steroid Biochem Mol Biol* 39:601–614.
- Mizukami S, Nishizuka M, Arai Y. 1983. Sexual difference in nuclear volume and its ontogeny in the rat amygdala. *Exp Neurol* 79:569–575.
- Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 13:1071–1082.
- Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z, Kuhn M, White RJ, Takaoka Y, Wolin L. 1975. The formation of estrogens by central neuroendocrine tissues. *Recent Prog Horm Res* 31:295–319.
- Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS. 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J Neurobiol* 39:569–578.
- Nishiyama A, Lin XH, Stallcup WB. 1995. Generation of truncated forms of the NG2 proteoglycan by cell surface proteolysis. *Mol Biol Cell* 6:1819–1832.
- Ormerod BK, Galea LA. 2001. Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. *Neuroscience* 102:369–379.
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19:8487–8497.
- Palmer TD, Takahashi J, Gage FH. 1997. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* 8:389–404.
- Paxinos G, Watson C. 1998. *The rat brain in stereotaxic coordinates*, 4th Edition. San Diego: Academic Press.
- Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J Neurosci* 21:6706–6717.
- Pfaff D, Keiner M. 1973. Atlas of estradiol-concentrating cells in the central nervous system of the female rat. *J Comp Neurol* 151:121–158.
- Romeo RD, Richardson HN, Sisk CL. 2002. Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. *Neurosci Biobehav Rev* 26:381–391.
- Roselli CE, Ellinwood WE, Resko JA. 1984. Regulation of brain aromatase activity in rats. *Endocrinology* 114:192–200.
- Roselli CE, Resko JA. 1987. The distribution and regulation of aromatase activity in the central nervous system. *Steroids* 50:495–508.
- Seaberg RM, van Der Kooy D. 2002. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci* 22:1784–1793.
- Shihabuddin LS, Horner PJ, Ray J, Gage FH. 2000. Adult Spinal Cord Stem Cells Generate Neurons after Transplantation in the Adult Dentate Gyrus. *J Neurosci* 20:8727–8735.
- Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E. 2001. Neurogenesis in the adult is involved in the formation of trace memories. *Nature* 410:372–376.
- Simerly RB. 1990. Hormonal control of neuropeptide gene expression in sexually dimorphic olfactory pathways. *Trends Neurosci* 13:104–110.
- Simerly RB, Chang C, Muramatsu M, Swanson LW. 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol* 294:76–95.
- Smith CM, Luskin MB. 1998. Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream. *Dev Dyn* 213:220–227.
- Smith MT, Pencea V, Wang Z, Luskin MB, Insel TR. 2001. Increased number of BrdU-labeled neurons in the rostral migratory stream of the estrous prairie vole. *Horm Behav* 39:11–21.
- Song H, Stevens C, Gage F. 2002. Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417:39–44.
- Steel E, Hutchison JB. 1987. The aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), blocks testosterone-induced olfactory behaviour in the hamster. *Physiol Behav* 39:141–145.

- Storey AE, Snow DT. 1990. Postimplantation pregnancy disruptions in meadow voles: relationship to variation in male sexual and aggressive behavior. *Physiol Behav* 47:19–25.
- Tanapat P, Hastings NB, Reeves AJ, Gould E. 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci* 19:5792–5801.
- van Praag H, Kempermann G, Gage FH. 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* 2:266–270.
- Vermeulen A. 1993. The male climacterium. *Ann Med* 25:531–534.
- Wang C, Alexander G, Berman N, Salehian B, Davidson T, McDonald V, Steiner B, Hull L, Callegari C, Swerdloff RS. 1996. Testosterone replacement therapy improves mood in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81:3578–3583.
- Wang Z, De Vries GJ. 1995. Androgen and estrogen effects on vasopressin messenger RNA expression in the medial amygdaloid nucleus in male and female rats. *J Neuroendocrinol* 7:827–831.
- Wang Z, Hulihan T, Insel TR. 1997. Sexual and social experience is associated with different patterns of behavior and neural activation in male prairie voles. *Brain Res* 767:321–332.
- Wolf OT, Kirschbaum C. 2002. Endogenous estradiol and testosterone levels are associated with cognitive performance in older women and men. *Horm Behav* 41:259–266.
- Wood RI. 1996. Estradiol, but not dihydrotestosterone, in the medial amygdala facilitates male hamster sex behavior. *Physiol Behav* 59:833–841.
- Wood RI, Brabec RK, Swann JM, Newman SW. 1992. Androgen and estrogen concentrating neurons in chemosensory pathways of the male Syrian hamster brain. *Brain Res* 596:89–98.
- Wood RI, Newman SW. 1995. The medial amygdaloid nucleus and medial preoptic area mediate steroidal control of sexual behavior in the male Syrian hamster. *Horm Behav* 29:338–353.