THE JOURNAL OF COMPARATIVE NEUROLOGY 481:252–265 (2005)

Ovarian Steroids Influence Cell Proliferation in the Dentate Gyrus of the Adult Female Rat in a Dose- and Time-Dependent Manner

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ABSTRACT

In previous work, we have demonstrated that cell proliferation in the adult hippocampal formation is regulated by estrogen under both natural and experimental conditions. To determine the extent to which this regulation is affected by the dose or schedule of hormone treatment, or progesterone administration, we examined the impact of different acute and chronic ovarian hormone replacement regimens on cell production using the S-phase marker bromodeoxyuridine. Additionally, we investigated the long-term impact of surgical ovarian hormone depletion on the capacity of estrogen to stimulate cell proliferation and the production of new cells that express either TuJ1 (a marker of neuronal phenotype) or glial fibrillary acidic protein (GFAP; a marker of astroglial phenotype). Acute treatment with a moderate, but not a low or a high, dose of estrogen rapidly increased cell proliferation in ovariectomized (OVX) animals, an effect that was reversed by the administration of progesterone. In contrast, OVX animals that were chronically replaced with either estrogen alone (continuous or cyclic) or estrogen plus progesterone (cyclic) did not exhibit an estrogen-induced increase in cell proliferation 3 weeks following the onset of hormone replacement. In animals that were subjected to a prolonged absence of ovarian hormones, acute treatment with the moderate dose of estrogen failed to stimulate cell proliferation, and a decrease in the number of new cells expressing a neuronal phenotype was evident. Collectively, these results indicate that a prolonged reduction in ovarian hormones results in 1) a diminished responsiveness to estrogen over time in this system and 2) a decrease in neuron production that is unlikely to be reversible by standard regimens of hormone replacement. J. Comp. Neurol. 481:252–265, 2005.

Indexing terms: estrogen; progesterone; dentate gyrus; hippocampal formation

The ovarian steroid estrogen is known to exert significant structural and functional effects on brain regions that are associated with cognition. One such region, the hippocampal formation, exhibits continued neuron production throughout adulthood, a process that occurs at a substantial rate in a wide range of species (for review see Hastings and Gould, 2000) and may be involved in certain types of learning and memory (Gould et al., 1999; Shors et al., 2001, 2002). In previous work, we have found that ovarian hormones are capable of influencing cell production in the adult hippocampal formation under both natural and experimental conditions. Under natural conditions, adult female rats exhibit an increase in the proliferation of cells in the dentate gyrus during proestrus (when ovarian hormone levels are highest) compared with estrus and diestrus that results in a transient increase in the number of new granule neurons. Consistent with this observation, short-term depletion of ovarian hormones via ovariectomy diminishes the number of proliferating cells, an effect that can be rapidly reversed by the administra-

Grant sponsor: National Institutes of Health; Grant number: MH59740 (to E.G.).

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Received 29 March 2004; Revised 13 July 2004; Accepted 11 August 2004
DOI 10.1002/cne.20385
Published online in Wiley InterScience (www.interscience.wiley.com).

tion of a single 10-μg injection of estradiol (Tanapat et al., 1999). Subsequent studies examining the effect of acute replacement with the same dose of estrogen following ovariectomy have likewise reported an increase in cell proliferation, which appears to then be followed by a suppression of cell proliferation (Ormerod et al., 2003; Banasr et al., 2004).

At present, little is known regarding the extent to which estrogen's effect on cell proliferation is dependent on either the dose or the regimen of hormone administration. Thus far, studies employing continuous-release hormone implants suggest that chronic estrogen replacement under such conditions does not persistently stimulate cell proliferation (Perez-Martin et al., 2003). Given that intact adult females typically experience cyclic increases in estrogen that are rapidly followed by an increase in progesterone, this observation raises the possibility that the repeated stimulation of cell proliferation by estrogen observed in intact adult females (Tanapat et al., 1999) requires a pulsatile pattern of administration, the presence of progesterone, or both.

In the present study, we sought to characterize some of the factors that are capable of influencing estrogen regulation of cell production in the dentate gyrus. In our first experiments, we examined the effect of subcutaneous injection with different doses of estradiol on cell proliferation in OVX animals as well as the impact of progesterone administration following injection with a dose of estradiol known to stimulate this process. Subsequently, we assessed the effect of a persistent reduction of ovarian hormones via ovariectomy on the ability of precursor cells to respond to acute estrogen treatment with an increase in proliferation. We also investigated whether such a prolonged period of ovarian hormone reduction is associated with an overall decrease in granule neuron production by comparing the numbers of new cells expressing a neuronal marker in OVX and sham-operated animals. To explore the possibility that estrogen's proliferative effect occurs via direct activation of precursor cells, we then examined whether dividing cells within the dentate gyrus express either estrogen receptor (ER) alpha or ER beta using immunohistochemical methods. Finally, to establish whether estrogen's effects on cell production are affected by temporal characteristics of hormone administration, we examined the numbers of proliferating cells in OVX animals that were chronically replaced with either estrogen alone (continuous or cyclic) or estrogen plus progesterone (cyclic).

MATERIALS AND METHODS

Animal care and treatments

Adult female (240–300 g) Sprague-Dawley rats from Taconic Farms (Germantown, NY) and the breeding colony at Princeton University were used in all experiments. Rats were housed in groups of three or four and maintained on a 12/12-hour light/dark cycle (lights on 7:00 AM) and provided with unlimited access to food and water.

Lavage

In experiments in which stage of estrous was determined, vaginal cytology was examined (Stockard and Papanicolaou, 1917; Shors et al., 1999) for a minimum of 10 days between 9:00 AM and 11:00 AM before treatment. The rat estrous cycle lasts 4 or 5 days and consists of three stages: proestrus, which lasts for 12 hours; estrus, which lasts for 36 hours; and diestrus, which lasts for either 48 or 72 hours (Long and Evans, 1922). In general, approximately 97% of the animals were judged to be cycling normally. Only regularly cycling animals were included in the experiments.

Surgery and hormone administration

Animals were anesthetized with Nembutal (40 mg/kg bw), and bilateral ovariectomy or sham ovariectomy was performed using aseptic procedures. After surgery, rats were warmed until recovery from anesthesia. For all experiments involving hormone treatment, animals were either 1) injected with estrogen or progesterone suspended in 100 μl sesame oil or with the same volume of vehicle alone or 2) implanted with estradiol or sham pellets. All hormone treatments were initiated 1 week following surgery and occurred at approximately 12:00 PM. This timing of hormone treatment was selected to be consistent with one that we have previously found to result in an estrogen-induced increase in cell proliferation (Tanapat et al., 1999). All of the animals within a single cage were subjected to the same treatment.

Euthanasia

For perfusion, all of the animals were deeply anesthetized at 4:00 PM with an overdose of sodium pentobarbital (Nembutal; 100 mg/kg bw) and then perfused transcardially with 4.0% paraformaldehyde in 0.1 M phosphate buffer. All animal experimentation was conducted in accordance with Princeton University guidelines and with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experimental design

Effects of acute ovarian hormone replacement on cell proliferation in the dentate gyrus

Experiment 1 (Fig. 1A). To determine whether estrogen stimulation of cell proliferation is dose-dependent, OVX females were subcutaneously injected with either sesame oil or one of the following doses of 17β-estradiol (E2): 1, 10, or 50 μg (n = 5–7 per group). Two hours later, all of the animals were injected with the S-phase marker bromodeoxyuridine (BrdU; 200 mg/kg bw i.p.) and perfused after a 2-hour survival. Previously, we have found that the 10-μg dose given subcutaneously is effective in stimulating cell proliferation (Tanapat et al., 1999). The 2-hour survival was chosen because it is sufficient for the uptake of BrdU into proliferating cells but not for mitosis or migration to occur (Nowakowski et al., 1989). The brains of these animals were processed immunohistochemically for the detection of BrdU incorporation. Previously, it has been shown that ovarian hormone manipulation is capable of altering blood–brain barrier permeability (Ziylan et al., 1990; Bishop and Simpkins, 1995; Oztas and Kaya, 1998), raising the possibility that the use of exogenous markers of proliferation might result in either a failure to detect or the false detection of differences in cell production. To address this issue, additional sections were also processed immunohistochemically for expression of the endogenous mitotic cell marker phosphohistone H3 (p-histone H3; Hendzel et al., 1997).

Experiment 2 (Fig. 1B). To investigate whether progesterone modulates the acute effects of estrogen on cell

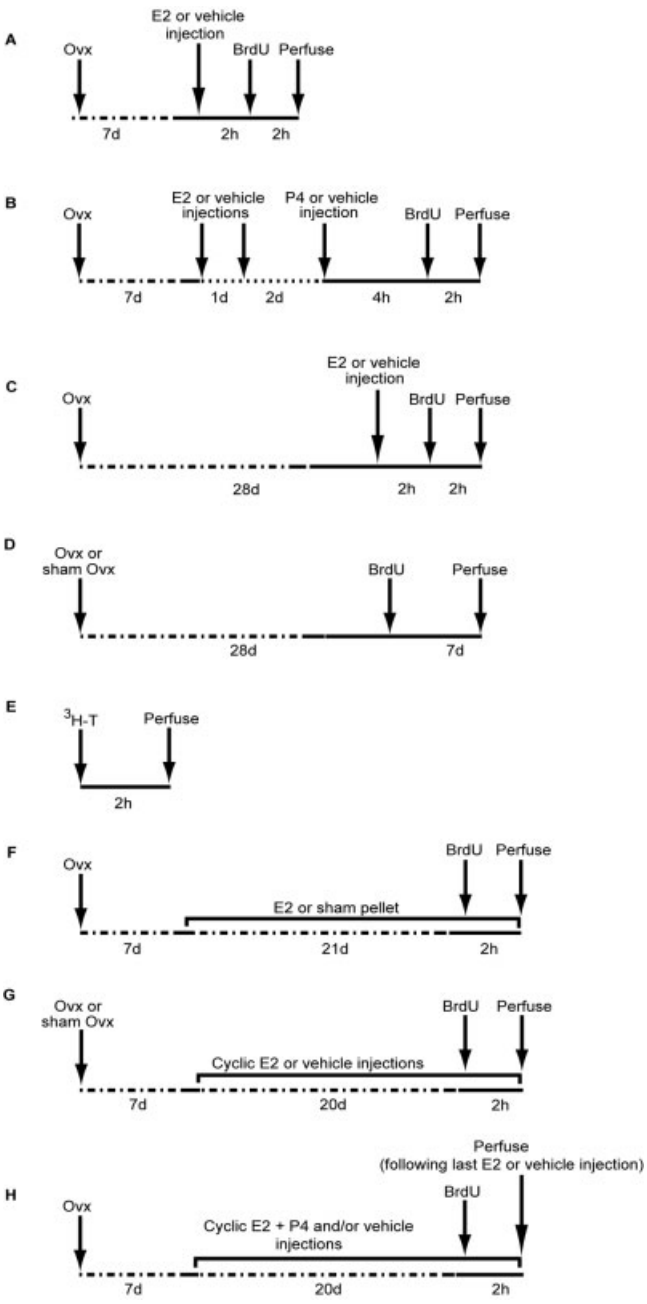

Fig. 1. **A–H:** Timelines indicating the onset of hormone replacement relative to surgical manipulation, the hormone replacement regimen used, and the timing of BrdU or ^{3}H-thymidine injection relative to hormone replacement in these experiments.

proliferation, OVX females were treated with one of the following regimens: 1) injection with E2 (10 μg s.c.) on Days 7 and 8 following surgery and progesterone (P4; 500 μg, s.c.) on Day 10, 2) injection with E2 on days 7 and 8 and sesame oil vehicle on Day 10, or 3) injection with vehicle on Days 7, 8, and 10 (n = 5–6 per group). These treatments were chosen because replacement with this regimen increases progesterone receptor expression and is sufficient to induce female sex behavior in OVX rats (McEwen, 1981). On Day 10, animals from each treatment group were injected with BrdU and transcardially perfused 2 hours later, and the brains were then processed for BrdU immunolabeling.

Effects of a persistent reduction of ovarian hormones on estrogen regulation of cell proliferation and neuron production in the dentate gyrus

Experiment 3 (Fig. 1C). To investigate whether adult female rats maintain the capacity to exhibit an estrogen-induced increase in cell proliferation following a persistent reduction of ovarian hormones, OVX females were injected with either E2 (10 μg, s.c.) or vehicle 4 weeks following surgery (n = 4 per group). Two hours later, all of the animals were injected with BrdU and perfused following a 2-hour survival. The brains of these animals were then processed for BrdU immunolabeling.

Experiment 4 (Fig. 1D). To determine whether a persistent reduction of ovarian hormones decreases the production of new hippocampal cells, counterbalanced cohorts comprising: 1) sham OVX females in diestrus (low estrogen), 2) sham OVX females in proestrus (high estrogen), and 3) OVX females were injected with BrdU between 28 and 35 days following surgery and perfused 1 week after BrdU injection (n = 5 per group). The brains of these animals were processed for BrdU immunolabeling alone, as well as for combined immunohistochemistry to detect BrdU incorporation and the expression of either glial fibrillary acidic protein [GFAP; an astroglial marker (Eng, 1985)] or TuJ1 [a marker of immature granule neurons (Geisert and Frankfurter, 1989)].

Expression of ER alpha and ER beta in the dentate gyrus

Experiment 5 (Fig. 1E). To determine whether classical ERs are expressed by progenitor cells in the dentate gyrus, adult female rats were injected with ^{3}H-thymidine (5.0 mCi/kg bw i.p.; Perkin-Elmer, Boston, MA; n = 5) and perfused after a 2-hour survival. The brains were then processed for ^{3}H-thymidine autoradiography combined with immunohistochemistry for the alpha and beta isoforms of the ER. Previously, we have found the antigen retrieval protocol for ER immunohistochemistry that results in the highest sensitivity of antibody binding to be incompatible with that of either p-histone H3 or BrdU. Therefore, the animals in this experiment were injected with ^{3}H-thymidine to identify proliferating cells.

Effects of different chronic ovarian hormone replacement regimens on cell proliferation in the dentate gyrus

Experiment 6 (Fig. 1F). To investigate whether continuous estrogen treatment persistently enhances cell proliferation in the dentate gyrus, OVX females were implanted 1 week following surgery with either a 0.25- or a 0.50-mg E2 pellet (30 days timed release; Innovative Research of America, Sarasota, FL) or a size-matched placebo pellet (n = 3–5 per group). Twenty-one days following pellet implantation, the animals were injected with BrdU and perfused after a 2-hour survival. The brains were then processed for both BrdU and p-histone H3 immunolabeling.

Experiment 7 (Fig. 1G). To determine whether cyclic estrogen treatment repeatedly stimulates cell proliferation in the dentate gyrus, OVX females began receiving injections of either E2 (10 μg s.c.) or oil vehicle every fourth day 1 week following surgery. At the same time, an additional group of sham OVX females began receiving

vehicle injections according to the same schedule (n = 8–9 per group). Two hours following the fifth E2 injection (20 days after the onset of hormone replacement), all of the animals were given a single injection of BrdU and perfused 2 hours later, and the brains were processed for both BrdU and p-histone H3 immunolabeling.

Experiment 8 (Fig. 1H). To establish whether cyclic treatment with both estrogen and progesterone results in repeated estrogen-induced stimulation of cell proliferation, OVX females began receiving weekly treatments 1 week following surgery according to one of the three hormone replacement schedules: 1) injection with E2 (10 μg, s.c.) on Days 7 and 8 following surgery and with P4 (500 μg, s.c.) on Day 10; 2) injection with E2 on Days 7 and 8 and with vehicle on Day 10; or 3) injection with vehicle on Days 7, 8, and 10. The first set of animals was subject to two cycles of hormone treatment (n = 4 per group), and the second set was subject to three cycles (n = 6 per group). Because this experiment was designed specifically to address the effect of cyclic ovarian hormone replacement on *estrogen*-induced changes in cell proliferation, all of the animals were given a single injection of BrdU after their last E2 or vehicle injection and perfused 2 hours later. The brains of animals in these two experiments were then processed for BrdU immunolabeling.

Trunk blood was collected at the time of perfusion, and the levels of circulating estradiol were determined via radioimmunoassay using a Coat-a-Count Kit (Diagnostics Product Corporation, Los Angeles, CA). For E2 kits, limit of detectibility = 8.0 pg/ml and cross-reactivity = 10.0% for estrone and <1.0% for most other steroid hormones.

Histological procedures

Forty-micrometer sections through the entire dentate gyrus of each brain were cut into a bath of 0.1 M phosphate-buffered saline (PBS; pH 7.2) with an oscillating tissue slicer. Unless otherwise indicated, all washes and incubations were performed in PBS for peroxidase immunohistochemistry and in 0.1 M Tris-buffered saline (TBS; pH 7.6) for fluorescence immunohistochemistry.

Peroxidase BrdU immunohistochemistry. For BrdU immunolabeling alone, sections were incubated in 0.1 M citric acid for 60 minutes at 75°C, rinsed, denatured in 2 N HCl for 60 minutes, rinsed, and incubated overnight at 4°C in mouse monoclonal antibody against BrdU (1:250 + 0.5% Tween-20; Novocastra, Newcastle Upon Tyne, United Kingdom). The sections were then rinsed, incubated in biotinylated mouse secondary antisera (1:200; Vector, Burlingame, CA) for 60 minutes, rinsed, incubated in avidin-biotin-horseradish peroxidase (AB; 1:100; Vector) for 60 minutes, rinsed, and reacted for 10 minutes in 0.01% diaminobenzidine (DAB) with 0.003% H_2O_2. The sections were then counterstained with cresyl violet, dehydrated, and coverslipped under Permount.

Combined fluorescence BrdU immunohistochemistry. Sections were denatured in 2 N HCl for 20–25 minutes, rinsed, and incubated overnight at 4°C in rat monoclonal antibody against BrdU (1:250 + 0.5% Tween-20; Accurate, Westbury, NY), rinsed, incubated in biotinylated rat secondary antisera (1:250; Chemicon, Temecula, CA) for 60 minutes, rinsed, incubated in streptavidin Alexa-568 (1:500; Molecular Probes, Eugene, OR), rinsed, and incubated in either mouse anti-TuJ1 (1:500; Chemicon) or goat anti-GFAP (1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 days at 4°C. After several rinses, sections were incubated in the appropriate secondary antisera conjugated to Alexa-488 (1:500; Molecular Probes) for 30 minutes. The sections were rinsed, mounted, dried, and then coverslipped under 25.0% glycerol in Tris-buffered saline (TBS).

p-Histone H3 immunohistochemistry

Sections were heated in 0.1 M citric acid at 80°C for 60 minutes, rinsed in PBS, incubated overnight in primary (1:100 plus 0.5% Tween-20; Upstate Biotechnology, Lake Placid, NY) at room temperature, rinsed, incubated in biotinylated rabbit secondary antisera for 60 minutes, rinsed, incubated in AB for 60 minutes, rinsed, and reacted for 5 minutes in DAB.

Combined ER alpha immunohistochemistry and ^{3}H-thymidine autoradiography

Sections were mounted onto slides, air dried, incubated in 0.1 M citric acid for 10 minutes at 90°C, and incubated for 2 nights at room temperature in mouse monoclonal antibody against ER alpha (1:500; Novocastra) or rabbit polyclonal against ER beta (1:100; Zymed, San Francisco, CA). After several rinses, the sections were then incubated in biotinylated mouse secondary antisera for 60 minutes, rinsed, incubated in AB for 60 minutes, rinsed, and reacted for 5 minutes in DAB. After an additional rinse, the slides were dipped in NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY) and stored in the dark for 3 weeks. The slides were then developed in Dektol developer (Electron Microscopy Sciences, Fort Washington, PA), rinsed, fixed in Polymax T fixer (Electron Microscopy Sciences), rinsed under running water for 20 minutes, counterstained for with cresyl violet, dehydrated, and coverslipped under Permount.

Data analysis

All of the slides were coded prior to quantitative analysis.

Peroxidase BrdU and p-histone H3 labeling

Stereological estimates of the total number of p-histone H3- and BrdU-labeled cells in the entire dentate gyrus were determined. BrdU-labeled cells on every twelfth section were counted at 1,000×, omitting cells located in the outermost plane of focus. After analysis, images were captured as tagged image format files in ImagePro Plus 3.0 (Media Cybernetics, Carlsbad, CA) and processed (adjustment of contrast and brightness) in Photoshop 5.0 (Adobe, San Jose, CA).

Combined fluorescence BrdU immunohistochemistry

For sections processed for combined BrdU immunohistochemistry, 50–60 cells per marker from anatomically matched sections were analyzed per brain. The numbers of BrdU-labeled cells in the granule cell layer (gcl) and subgranular zone (sgz) that were immunoreactive or nonimmunoreactive for TuJ1 or GFAP were determined with a confocal laser scanning microscope (Zeiss Axiovert 510 LSM), and the data expressed as percentages. Z-sectioning at 1-μm intervals was performed for analysis. For figures, optical stacks of 3–12 images were produced, exported as Photoshop

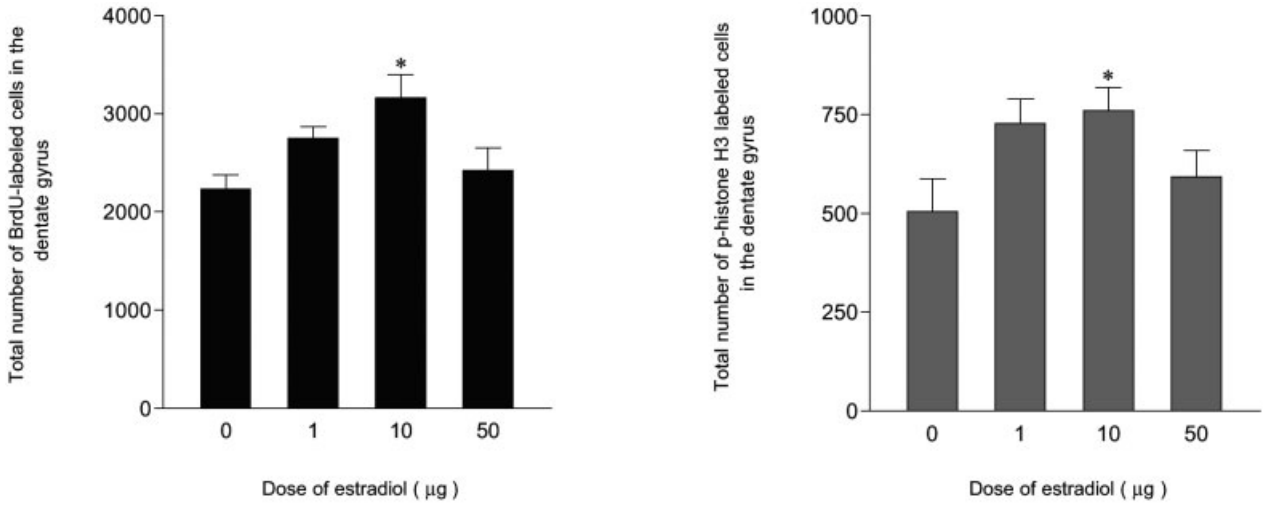

Fig. 2. Stereological estimates of the total number of BrdU-labeled cells (**left**) and p-histone H3-labeled cells (**right**) in the dentate gyrus of OVX adult rats that were replaced with either vehicle or 1, 10, or 50 μg of 17β-estradiol. Replacement with 10 μg, but not 1 μg or 50 μg, estradiol resulted in a significant increase in the numbers of BrdU- and p-histone H3-labeled cells in the dentate gyrus of adult female rats compared with OVX controls. Bars represent mean ± SEM, each obtained from five to seven animals. *$P < 0.05$ vs. vehicle-treated group mean.

files, and then processed in Photoshop 5.0 to optimize contrast and brightness.

^{3}H-thymidine autoradiography

For brains that were processed for combined ER immunohistochemistry and ^{3}H-thymidine autoradiography, every twelfth section through the extent of the dentate gyrus from each brain was analyzed for the presence of ^{3}H-thymidine-labeled cells that were immunoreactive for either ER alpha or ER beta. Images were obtained as described above for peroxidase BrdU and p-histone H3 immunohistochemistry.

Statistical analysis

Total cell counts were analyzed by using Student's *t*-tests or one-way ANOVA. Unplanned comparisons of means were carried out by using the Tukey HSD method for groups with equal sample sizes or the Spjotvoll-Stoline adjustment to the Tukey method for unequal sample sizes (Sokal and Rohlf, 1997). For comparisons that are statistically significant, the probability (alpha = 0.05) that a difference between a pair of means exceeds the minimum significant range is reported. The percentages of double-labeled cells were analyzed with distribution-free methods: the Kruskal-Wallis ANOVA by ranks followed by pairwise comparisons by the Mann-Whitney U test.

RESULTS

Effect of acute estrogen replacement at different doses on cell proliferation in the dentate gyrus

Analysis of the numbers of BrdU-labeled cells indicated that acute replacement with estradiol 1 week following ovariectomy stimulated cell proliferation in a manner that was dose-dependent [$F(3,16) = 4.12$, $P = 0.024$]. Injection of OVX rats with 10 μg, but not 1 or 50 μg, of estradiol significantly increased the numbers of BrdU-labeled cells observed in the dentate gyrus 2 hours following BrdU injection ($P = 0.023$; Fig. 2A). Likewise, analysis of the numbers of p-histone H3-labeled cells also revealed a dose-dependent effect of estradiol treatment [$F(3,20) = 3.93$, $P = 0.026$]. The numbers of p-histone H3-labeled cells were also increased in animals that were injected with 10 μg of estradiol compared with vehicle-treated controls ($P = 0.041$; Fig. 2B).

Both BrdU-labeled and p-histone H3-labeled cells were occasionally observed in the granule cell layer but were found predominantly in the sgz and hilus in all of the groups. p-Histone H3-labeled cells exhibited the morphological characteristics of cells undergoing mitosis (Fig. 3A–D) and displayed a distribution similar to that observed with BrdU labeling. Additionally, statistical analysis indicated that the number of p-histone H3-labeled cells correlated well with the number of BrdU-labeled cells ($r = 0.88$, $P < 0.0001$; Fig. 4).[1] Radioimmunoassay confirmed that increasing doses resulted in increasingly elevated estradiol levels in hormone-replaced animals compared with vehicle-treated controls (Table 1).

Influence of progesterone on the effect of acute estrogen treatment on cell proliferation in the dentate gyrus

Acute ovarian hormone treatment significantly altered the numbers of BrdU-labeled cells in OVX animals [$F(2,18) = 5.38$, $P = 0.022$]. On Day 10 following surgery,

[1]The overall greater numbers of BrdU-labeled cells compared with p-histone H3-labeled cells are not unexpected given that the length of S phase is significantly greater than that of M phase (Cameron and McKay, 2001).

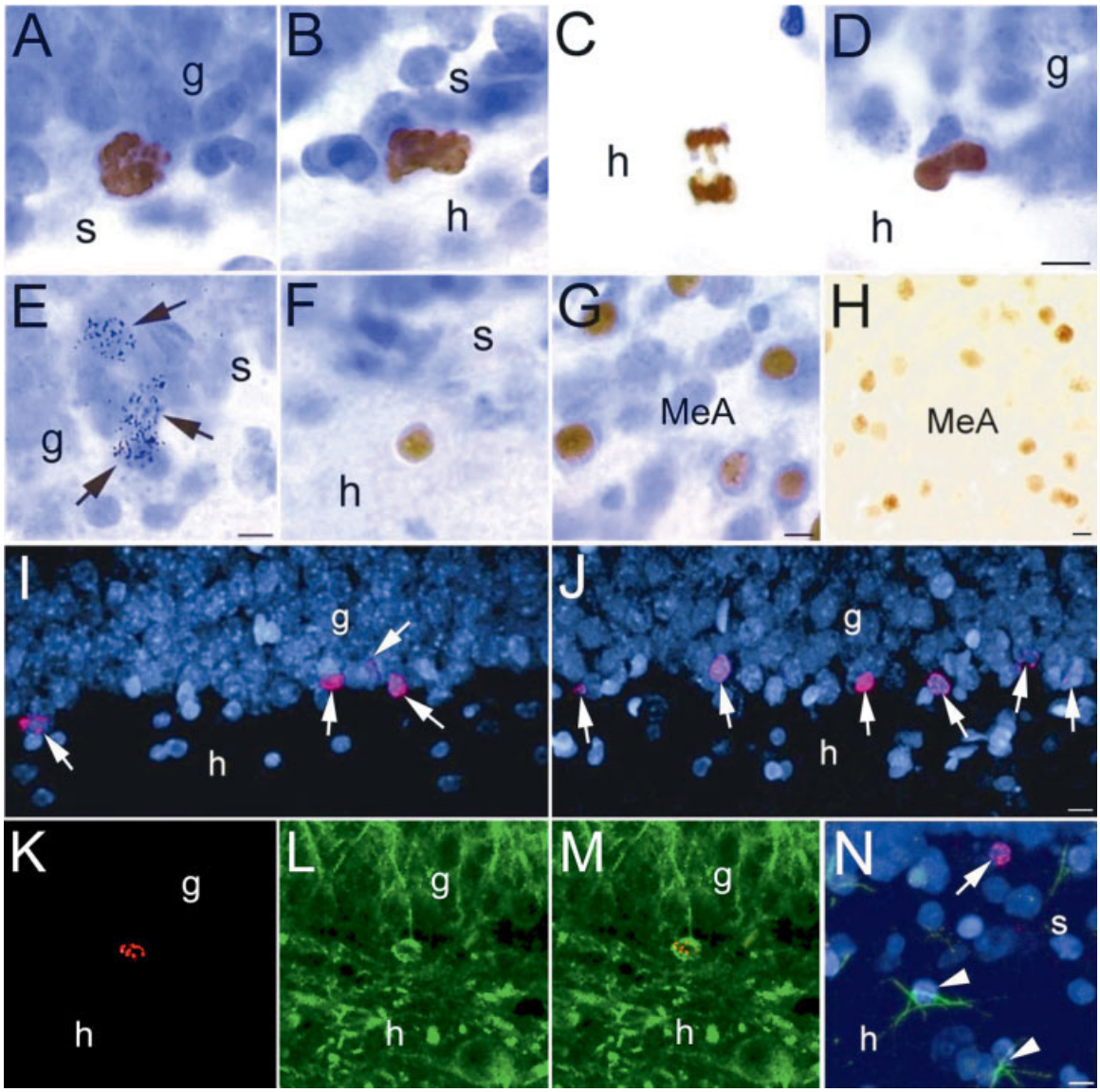

Fig. 3. Photomicrographs of adult-generated cells in the dentate gyrus of adult female rats. Acute replacement with estrogen 1 week following ovariectomy stimulated cell proliferation in a manner that was dose dependent; injection of OVX rats with 10 μg, but not 1 μg or 50 μg, estradiol significantly increased the numbers of BrdU- and p-histone H3-labeled (A–D) cells observed in the dentate gyrus. P-histone H3-labeled cells exhibited the morphological characteristics of cells undergoing mitosis (**A:** late prophase; **B:** metaphase; **C:** anaphase; **D:** telophase) and displayed a distribution similar to that observed with BrdU labeling. The proliferative effects of estrogen do not appear to occur via the direct activation of granule cell precursors. Although ER alpha-immunoreactive cells (**F**) were observed in both the hilus and the sgz of all females examined, none of these cells was colabeled with ^{3}H-thymidine (**E**, arrows) 2 hours following injection. Both ER alpha (**G**)- and ER beta (**H**)-immunoreactive cells were observed in subcortical regions, including the medial amygdala. However, no cells that were immunoreactive for ER beta were observed in the dentate gyrus. One week following BrdU injection, sham OVX females that were injected during proestrus (**I**) had a greater number of BrdU-labeled cells (arrows) compared with sham females injected during diestrus and OVX females (**J**) that were injected at the same time. In all of these animals, ~75% of BrdU-labeled cells (K–N, red) expressed the marker of immature granule neurons TuJ1 (**K–M**, green) whereas ~10% expressed the marker of astroglia GFAP (**N**, green). K–M depict a cell that is immunoreactive for both BrdU and TuJ1, showing the cell as it appears when imaged to visualize BrdU alone (K), TuJ1 alone (L), and both BrdU and TuJ1 (M). Arrow in N indicates a BrdU-labeled cell that is not colabeled with GFAP. Arrowheads in N indicate cells that are immunolabeled for GFAP alone. g, Granule cell layer; s, subgranular zone; h, hilus; MeA, medial amygdala. Scale bars = 10 μm in D (applies to A–D); 10 μm in E; 10 μm in G (applies to F,G); 10 μm in H; 10 μm in J (applies to I,J); 10 μm in N (applies to K–N).

OVX animals that were injected with 10 μg estradiol demonstrated a significant increase in the numbers of BrdU-labeled cells in the dentate gyrus compared with OVX animals receiving only vehicle ($P = 0.035$; Fig. 5). However, estradiol-treated animals that were injected with 500 μg progesterone on Day 10 exhibited numbers of BrdU-labeled cells that were similar to those of vehicle-treated OVX females.

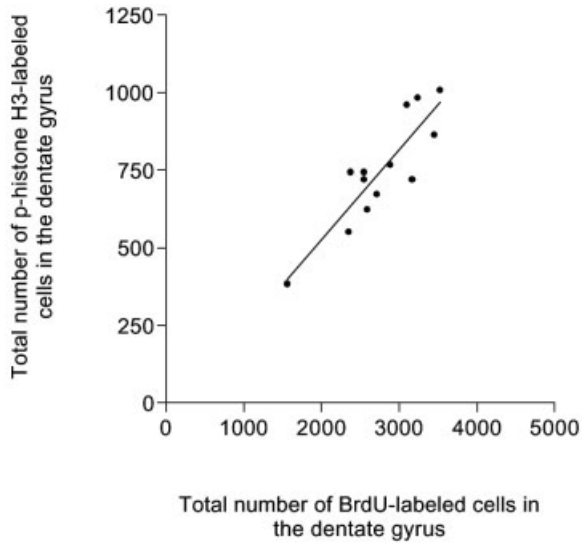

Fig. 4. Statistical analysis revealed a significant correlation between the numbers of BrdU- and p-histone H3-labeled cells in the dentate gyrus of adult female rats that were OVX and replaced with vehicle or 1, 10, or 50 μg of 17β-estradiol, injected with BrdU, and perfused following a 2-hour survival ($r = 0.88$, $P < 0.0001$).

TABLE 1. Circulating Levels of Estradiol in OVX Rats That Were Injected Subcutaneously with Vehicle or 1, 10, or 50 μg of 17β-Estradiol 1 Week Following Surgery[1]

Dose of estradiol (μg)	Serum estradiol level (pg/ml)
0.0	n.d.
1.0	12.0 ± 3.39
10.0	110.5 ± 9.97
50.0	532.9 ± 67.27

[1]Radioimmunoassay confirmed that increasing doses resulted in increasingly elevated estradiol levels in hormone-replaced animals compared with OVX controls.

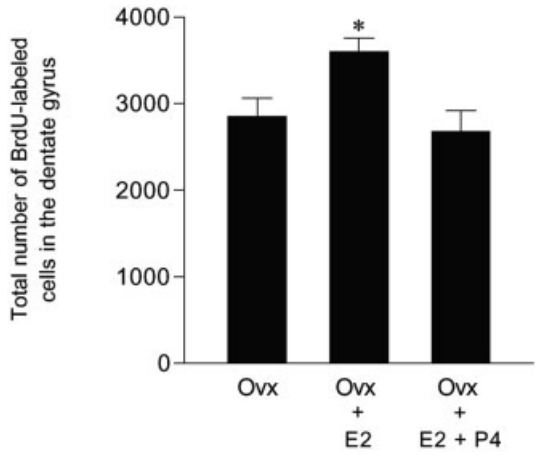

Fig. 5. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of OVX adult rats that were treated with estrogen and progesterone, estrogen, or vehicle. Treatment with progesterone 48 hours following the last of two daily injections with estradiol rapidly reversed an estrogen-induced increase in cell proliferation. Bars represent mean ± SEM, each obtained from five or six animals. *$P < 0.05$ vs. other means.

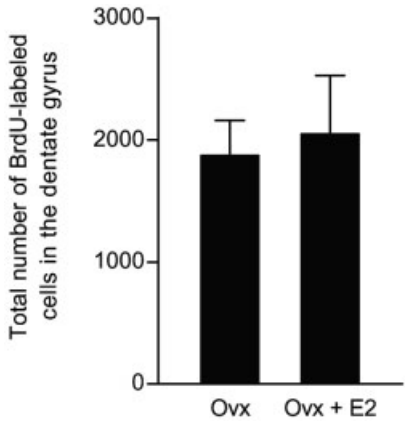

Fig. 6. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of OVX adult rats that were injected with either 10 μg E2 or vehicle 4 weeks following surgery. Two hours after BrdU injection, no differences were detected in the numbers of BrdU-labeled cells between estrogen- and vehicle-replaced animals. Bars represent mean ± SEM, each obtained from four animals.

Effect of a persistent reduction of ovarian hormones on estrogen regulation of cell proliferation and neuron production in the dentate gyrus

In contrast to observations 1 week following ovariectomy (Experiment 1), no differences in the number of BrdU-labeled cells were detected between females that received a single 10-μg injection of estradiol and those that received vehicle injections 4 weeks following surgery [$t(1,5) = 0.334$, $P = 0.752$] (Fig. 6). Likewise, no differences were detected in the number of p-histone H3-labeled cells between groups [$t(1,8) = 0.352$, $P = 0.734$]. As in animals that underwent ovariectomy 1 week prior to hormone treatment, labeled cells were occasionally observed in the granule cell layer but were found predominantly in the sgz and hilus in both groups.

A significant difference in the number of BrdU-labeled cells was detected across sham OVX and OVX females that were injected with BrdU 4 weeks following surgery and allowed to survive for an additional 1 week [$F(2,10) = 4.995$, $P = 0.031$]. Sham OVX females that were injected with BrdU during proestrus demonstrated a greater number of BrdU-labeled cells in the dentate gyrus 1 week after the injection compared with sham OVX females injected during diestrus ($P = 0.045$), whereas OVX females did not (Fig. 7). No differences were detected in the distribution of cells throughout the dentate gyrus across groups at this time point; BrdU-labeled cells were observed primarily in the sgz and in the deep aspect of the gcl and less frequently in the hilus (Fig. 3I,J). Additionally, no differences were detected across groups in the percentage of BrdU-labeled cells that expressed either TuJ1 ($x_{\text{sham OVX Diestrus}} = 74.8 \pm 1.45$, $x_{\text{sham OVX Proestrus}} = 77.4 \pm 0.71$, $x_{\text{OVX}} = 72.5 \pm 0.74$; Fig. 3K–M) or GFAP ($x_{\text{sham OVX Diestrus}} = 11.1 \pm 0.6$, $x_{\text{sham OVX Proestrus}} = 9.7 \pm 1.17$, $x_{\text{OVX}} = 12.1 \pm 2.25$; Fig. 3N).

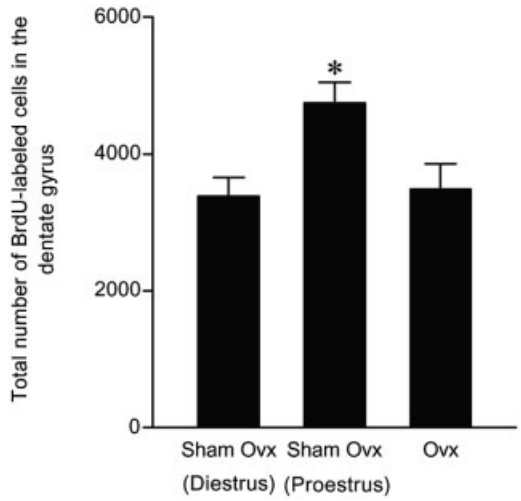

Fig. 7. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of sham OVX and OVX adult rats that were injected with BrdU between 28 and 35 days following surgery. The mean numbers of BrdU-labeled cells following the 1–2-week survival were approximately 50% greater than those observed following a 2-hour survival (experiments 1–3, 6–8), an increase that is consistent with our previous observations of the levels of BrdU labeling in adult females with increasing postinjection time (Tanapat et al., 1999). One week following BrdU injection, sham-operated females that were in proestrus at the time of injection had a greater number of BrdU-labeled cells compared with those injected during diestrus as well as similarly injected OVX animals. Bars represent mean ± SEM, each obtained from five animals. $^{*}P < 0.05$.

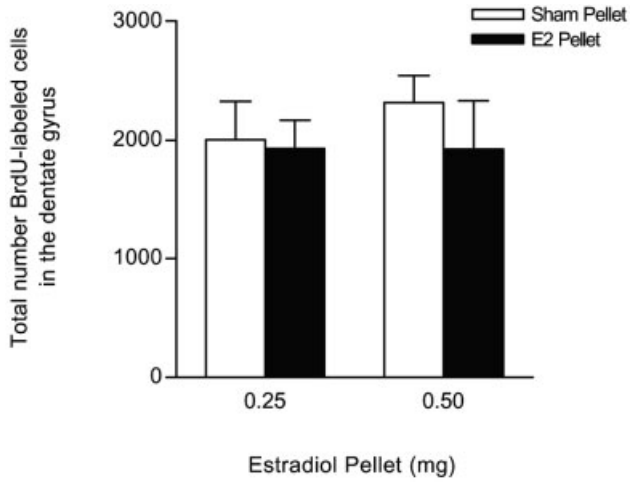

Fig. 8. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of OVX rats that were implanted with either 0.25- or 0.50-mg E2 pellets or size-matched sham pellets for 3 weeks. Two hours following BrdU injection, no differences were detected between E2-treated animals and sham-replaced controls. Bars represent mean ± SEM; n = 3–5 per group.

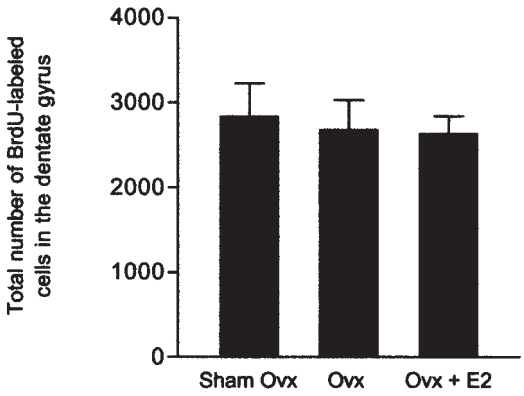

Fig. 9. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of animals that were OVX and injected every 4 days with either 10 μg E2 or vehicle or sham-operated and injected with vehicle every 4 days for 3 weeks. Two hours following BrdU injection, no differences in the number of proliferating cells were detected across groups. Bars represent mean ± SEM; n = 8–9 per group.

Expression of ER alpha and beta in the dentate gyrus

Consistent with the results of past studies, cells that were immunoreactive for ER alpha were observed in a number of regions, including the ventromedial hypothalamus, preoptic area, and medial amygdala (Fig. 3H) as well as in the hilus and sgz (Fig. 3F) of the dentate gyrus (Weiland et al., 1997; Mufson et al., 1999; Hart et al., 2001; Mitra et al., 2003). However, none of the ER alpha-immunolabeled cells detected in either the hilus or the sgz was colabeled for ^{3}H-thymidine (Fig. 3E) 2 hours following injection. Also as previously reported, ER beta-immunoreactive cells were observed in a number of brain regions, including the paraventricular and supraoptic nuclei of the hypothalamus and the medial amygdala (Fig. 3G), but not in the dentate gyrus (Alves et al., 1998a; Shughrue and Merchenthaler, 2001; Greco et al., 2001; Mitra et al., 2003).

Effects of continuous and cyclic ovarian hormone replacement regimens on cell proliferation in the dentate gyrus

Continuous replacement with estradiol for 3 weeks did not persistently increase the rate of cell proliferation in the dentate gyrus. No differences in the numbers of BrdU-labeled cells were detected between E2- and vehicle-treated OVX animals 3 weeks after implantation with either a 0.25-mg [$t(1,7) = 0.190$, $P = 0.855$] (Fig. 8) or a 0.50-mg pellet [$F(1,5) = 0.713$, $P = 0.527$] (Fig. 8). These results were consistent with our observations of the numbers of p-histone H3-labeled cells, which also failed to demonstrate an effect of estrogen treatment with either the 0.25-mg dose [$t(1,5) = 0.357$, $P = 0.735$] or the 0.50-mg dose [$t(1,4) = 0.822$, $P = 0.457$]. In this experiment, statistical analysis indicated that the number of p-histone H3-labeled cells did not correlate with the number of BrdU-labeled cells.

Similarly, cyclic injections with 10 μg estradiol alone every 4 days for 3 weeks did not result in repeated stimulation of cell proliferation in the dentate gyrus. No differences in the numbers of BrdU-labeled cells were detected across the sham OVX, OVX, or OVX + E2 groups in animals perfused 3 weeks after the onset of estrogen replacement [$F(2,24) = 0.343$ $P = 0.713$] (Fig. 9). Similar results were also observed with regard to the numbers of p-histone H3-labeled cells [$F(2,18) = 1.091$ $P = 0.357$].

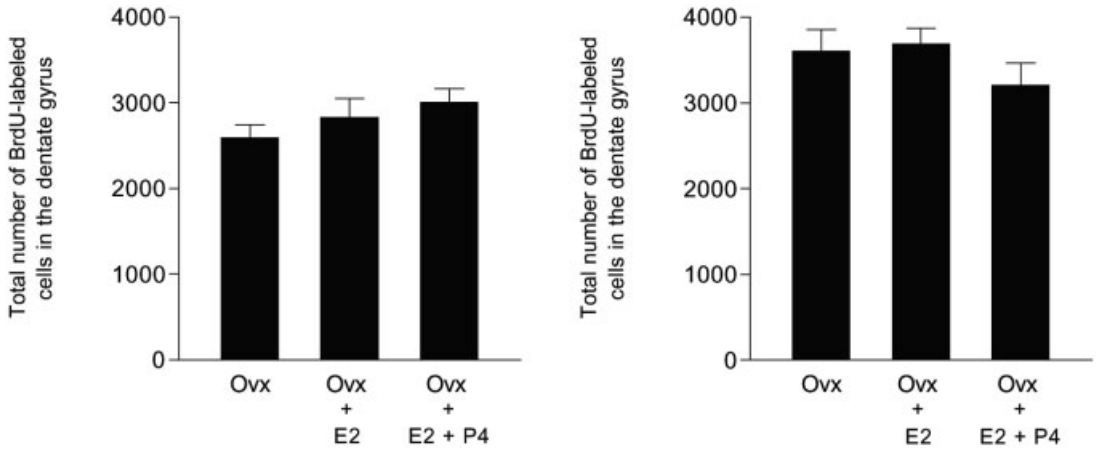

Fig. 10. Stereological estimates of the total number of BrdU-labeled cells in the dentate gyrus of OVX adult rats that were replaced cyclically with E2 + P4, E2, or vehicle for either 2 (**left**) or 3 (**right**) weeks. Two hours following BrdU injection, no differences were detected in the numbers of BrdU-labeled cells at either time point. Bars represent mean ± SEM, each obtained from four or five animals.

In animals that were cyclically replaced with two 10-μg estradiol injections over 2 days, followed by a 500-μg progesterone injection 2 days later, an estradiol-induced increase in the proliferation of granule cell precursors was not observed, independent of whether progesterone was also administered. Four hours following the last E2 or vehicle injection, no differences in the numbers of BrdU-labeled cells were observed in animals that had been cyclically injected with E2 + P4, E2, or vehicle alone for either two [$F(2,6) = 1.080$, $P = 0.399$] (Fig. 10A) or three [$F(2,10) = 1.201$, $P = 0.340$] (Fig. 10B) hormone cycles.

Radioimmunoassay confirmed that estradiol levels were elevated in hormone-replaced animals compared with OVX controls in all experiments. With regard to sham animals, circulating estradiol levels were, on average, comparable to levels in animals in diestrus (Shors et al., 1999).

DISCUSSION

Our findings indicate that the effects of estrogen on cell proliferation in the dentate gyrus are dependent on the dose and duration of hormone treatment and can be modulated by progesterone. Acute treatment with a moderate, but not a low or high, dose of estrogen rapidly increased cell proliferation in OVX animals, an effect that was reversed by progesterone administration. In contrast, OVX animals that were chronically replaced with either estrogen alone (continuous or cyclic) or estrogen plus progesterone (cyclic) did not exhibit an estrogen-induced increase in cell proliferation 3 weeks following the onset of hormone replacement. This finding suggests that a decrease in neuron production observed with long-term ovarian steroid reduction is not likely to be reversible by standard regimens of hormone administration.

Estrogen effects are dose-dependent

Consistent with our previous observations, we found that the administration of 10 μg, but not 1 or 50 μg, of estradiol rapidly stimulated an increase in the numbers of proliferating cells in the dentate gyrus of OVX animals (Tanapat et al., 1999). This is generally in agreement with other reports that estrogen is capable of altering numerous physiological and behavioral measures at low to moderate, but not relatively high, doses of hormone (Diaz-Veliz et al., 1991; Moreira et al., 1997; Rusa et al., 1999). Although the mechanisms underlying such dose-dependent effects of estrogen have yet to be fully elucidated, these phenomena are likely to be, at least in part, due to ER down-regulation (Medlock et al., 1991a,b; Lauber et al., 1991a).

When considering the results presented in this study, it should be noted that subcutaneous estradiol injection following ovariectomy OVX results in a hormonal profile that differs greatly from the one observed under natural conditions in an intact nonpregnant adult female rat. Whereas intact females exhibit circulating estradiol levels ranging from undetectable to approximately 50 pg/ml (Shors et al., 1999), OVX females that are estradiol replaced via injection or subcutaneous implantation will initially exhibit a nonphysiological peak in hormone level regardless of the dose administered (Medlock et al., 1991a). Thus, it becomes difficult to make direct comparisons between our present observations and processes typical of intact animals. However, we have previously found that intact adult females exhibit cyclic increases in cell proliferation when estrogen levels are high (a change that underlies a proestrus-associated increase in the production of immature hippocampal neurons), indicating that estrogen-stimulated cell proliferation is indeed a physiologically relevant phenomenon. Although we did not directly compare the level of cell proliferation in estradiol-replaced OVX animals with levels observed across the normal estrous cycle, our work collectively indicates a level in OVX animals that is comparable to diestrus and estrus levels in the absence of hormone replacement and comparable to proestrus levels following a single injection with 10 μg estradiol (experiment 1; Tanapat et al., 1999). Nevertheless, the conditions required to restore normal patterns of cell proliferation and neuron production in OVX animals over extended periods are unknown. To address this question, the future application of more appro-

priate methods of hormone replacement that might be capable of faithfully reproducing normal patterns of ovarian hormone level (e.g., using timed-release capsules that are capable of delivering variable doses of hormone) will likely be required.

Progesterone reverses the acute effect of estrogen on cell proliferation

Our data indicate that progesterone administration rapidly reverses an increase in cell proliferation in the dentate gyrus observed following acute replacement with estrogen. This observation, which is in agreement with studies of other cell systems (Tachi et al., 1972; Moutsatsou and Sekeris, 1997), suggests that the opposing influences of estrogen and progesterone may be responsible for the fluctuation in cell proliferation normally observed across the estrous cycle (Tanapat et al., 1999). This type of antagonism between estrogen and progesterone has been demonstrated in other physiological and behavioral systems (Woolley and McEwen, 1993) and may play a role in maintaining estrogen responsiveness of precursor cells in the intact animal (Morin, 1977; Satou and Yamanouchi, 1996; Zhu et al., 1999; Numan et al., 1999). Although the inclusion of progesterone in a chronic (cyclic) hormone replacement regimen failed to preserve estrogen responsiveness in the present study, the possibility that its influence contributes to the pattern of cell proliferation observed in intact females cannot be completely ruled out.

Currently, it is not known whether progesterone might be capable of exerting an inhibitory effect on the proliferation of hippocampal precursor cells in the absence of estrogen priming. In the present work, the effects of progesterone treatment alone on cell production were not assessed because the experiments were designed in an attempt to replicate a naturally occurring condition, i.e., a proestrus-associated increase in cell proliferation. However, our data indicating that progesterone is capable of reversing the stimulatory effect of estrogen on cell production raises the possibility of an independent action of progesterone. Indeed, such a finding would be consistent with previous reports indicating that treatment with progesterone can inhibit cell proliferation in other cell systems, including the adult subependymal layer (Moutsatsou and Sekeris, 1997; Backlin et al., 2003; Giachino et al., 2004).

Potential mechanisms of estrogen-induced increases in cell proliferation

Presently, it remains unclear whether the effects of estrogen on cell proliferation in the dentate gyrus are mediated via direct activation of precursor cells within the dentate gyrus. By using two previously well-characterized antibodies (Mufson et al., 1999; Hart et al., 2001; Shughrue and Merchenthaler, 2001; Kritzer, 2002), we found that dividing cells in the dentate gyrus do not express either of the classical ERs, alpha or beta. However, previous reports regarding the distribution of ER protein, mRNA, and binding activity within the brain have been highly conflicting (Pfaff and Keiner, 1973; Loy et al., 1988; Shughrue et al., 1997; Shughrue and Merchenthaler, 2000a,b, 2001; Hart et al., 2001). A potential explanation for these inconsistencies might involve the presence of other ER variants, which may, in turn, be responsible for mediating the effects of estrogen on hippocampal precursor cells. Perhaps related to this possibility, the presence of nonnuclear ERs in several different cell populations of the hippocampal formation has recently been demonstrated (Milner et al., 2001). Although these receptors appear to be present primarily in cells expressing a differentiated phenotype, their identification raises questions regarding whether precursor cells may express nonclassical estrogen receptors as well.

Considerable ambiguity also exists with regard to whether estrogen-induced changes in cell proliferation are likely to be mediated via classical ER actions or the more recently recognized rapid nongenomic effects of estrogen. Our data indicate that an increase in cell proliferation may be observed as early as 4 hours following a single estrogen injection and as late as 48 hours following two daily injections. Although the duration of the effect on cell proliferation suggests that changes in gene transcription are likely to be involved, it is also conceivable that the initial increase is mediated by nongenomic mechanisms. An example of this type of dynamic has recently been described for the estrogen-induced increase in spine density observed in the CA1 pyramidal region. This phenomenon, which occurs within a similar time frame, is characterized by an initial phase resulting from a rapid estrogen effect, followed by a secondary phase that can be blocked by inhibitors of the classical ERs (Rudick and Woolley, 2003).

Alternatively, there is the possibility that estrogen modulates cell proliferation indirectly by acting on cell populations that provide afferent input to the dentate gyrus. Although no studies to date have investigated the neuroanatomic projections that are involved in estrogen stimulation of cell proliferation within the dentate gyrus, both ER alpha and ER beta have been found to be expressed by cell populations within the entorhinal cortex (Kritzer, 2002; Shima et al., 2003) as well as the dorsal raphe nucleus (DRN; Alves et al., 1998b; Mitra et al., 2003; Warembourg and Leroy, 2004). With regard to the latter, a previous study has demonstrated that estrogen stimulation of precursor cells can be mediated indirectly via the neurotransmitter serotonin (Banasr et al., 2004). However, the serotonergic cells of the DRN that project directly to the hippocampal formation do not appear to express ER alpha (Alves et al., 1998b), and it is not yet known whether they express ER beta. Thus, the presence of estrogen-responsive cells with the potential to act directly on precursor cells in this brain region remains to be established.

With regard to the cellular events that underlie estrogen's mitogenic effects, studies in nonneuronal systems indicate that estrogen is capable of stimulating mitosis by decreasing the length of G1 as well as via regulation of the G1/S transition (Sutherland et al., 1983; Geum et al., 1997; Hong et al., 1998). Studies that have examined estrogen-induced changes in the cell cycle of rodent uterine (Bonapace et al., 1996) and mammary gland (Epifanova, 1966) cells suggest that both mechanisms are likely to play an important role. Although our present studies do not directly address this issue, our finding that estrogen stimulates an increase in the number of mitotic cells within 4 hours following estrogen administration raises the additional possibility of a mechanism of regulation that acts at the G2/M transition of the cell cycle.

Effects of chronic hormone replacement pattern on estrogen stimulation of cell proliferation

The results of the present study indicate that the increase in cell proliferation observed with acute estrogen treatment diminishes over time with chronic hormone exposure. This finding parallels previous observations of estrogen's effects on granule cell spine density in aged female rats; following prolonged ovarian hormone deprivation, short-term replacement with estradiol increases spine density levels, whereas long-term replacement does not (Miranda et al., 1999). The loss of an estrogen effect in these cases is perhaps not surprising given that numerous studies have reported persistent ER down-regulation under conditions of chronic estrogen exposure (Lauber et al., 1991b; Brown et al., 1993). However, ER replenishment has been reported to occur within a period of hours following both estradiol injection and estradiol implant withdrawal (Gomez-Benitez and Diaz-Chico, 1985; Bergman et al., 1987; Medlock et al., 1991a,b), suggesting that cyclic regimens such as the two employed in the present study should provide sufficient time for recovery of responsiveness.

As is the case with a single subcutaneous injection, the extent to which observations deriving from chronic hormone manipulations are relevant to naturally occurring conditions is difficult to determine. Implicit in the observation that proestrus is characterized by increases in the numbers of proliferating cells is the fact that precursor cells are capable of repeated response to estrogen input. However, we did not observe such an effect of estrogen even under conditions of cyclic ovarian hormone exposure. A possible explanation for this may involve the timing and dose of cyclic hormone replacement, which were chosen primarily based on data regarding estrogen regulation of cell proliferation and not the degree to which they mimic the diestrus and proestrus environments. Thus, the lack of an estrogen effect might have simply been due to the fact that subcutaneous injections do not result in a hormonal profile sufficiently similar to that of an intact adult female rat.

Alternatively, it may be that the stress associated with repeated subcutaneous injections was responsible for the lack of an estrogen effect on cell proliferation. Prior studies indicate that stressful experiences significantly decrease the proliferation of hippocampal cells (Gould et al., 1997, 1998; Tanapat et al., 2001) and, moreover, that this effect may be additive under conditions of chronic stress (Czeh et al., 2002). However, a previous report suggests that exposure to fox odor, an experience that has consistently been shown to suppress cell proliferation in adult male rats (Tanapat et al., 2001; Falconer and Galea, 2003), does not have a similar impact on adult female rats (Falconer and Galea, 2003). Nevertheless, the effects of the duration or type of stress on cell proliferation in the female have yet to be fully characterized. Thus, it remains possible that stress might have altered the effects of estrogen in the current study.

It should be noted that our data regarding the impact of a prolonged reduction in ovarian hormones also raises the possibility that a fundamental change occurs over time within the system that diminishes the ability of estrogen to stimulate cell proliferation. This may be due to the supraphysiological peaks of circulating estradiol levels. However, changes related to other aspects of hormone treatment are likely to occur as well. For example, studies have shown that estradiol-implanted animals will exhibit spontaneous daily surges of luteinizing hormone (Legan and Karsch, 1975; Freeman et al., 1976; Bethea and Weiner, 1983). These surges eventually disappear by 10 days following implantation, signifying that changes in sensitivity occur even following the initial stages of hormone replacement. At present, the extent to which normal physiological conditions might be maintained by the administration of a more physiologically relevant replacement regimen remains to be determined.

Potential functional implications of estrogen regulation of adult hippocampal cell production

Ovarian hormones have been shown to mediate dramatic changes in physiology that prepare the female for reproduction. However, despite the fact that the development of sexual receptivity, induction of the first ovulation, pregnancy, and maternal behavior have been widely studied, little is known about the mechanisms underlying the dramatic physiological changes and reorganization of behavior that accompany these events. Some studies have linked the hippocampal formation to regulation of the onset of female puberty (Terasawa and Timiras, 1968; Docke, 1974, 1977) and expression of maternal behavior (Kimble et al., 1967). If the hippocampal formation does participate in these events, then estrogen-regulated hippocampal cell production presents a potential physiological substrate for the behavioral changes observed during these periods.

In addition to mediating physiological and behavioral processes that are associated with reproduction, estrogen has also been reported to influence cognition. Presently, the nature of estrogen's impact on cognitive processes associated with the hippocampal formation remains somewhat unclear. Whereas several studies have shown that treatment with estrogen enhances performance on hippocampal-associated tasks, others have reported either no change or even a detrimental effect of estrogen (for review see Dohanich, 2002; cf. Sandstrom and Williams, 2001, 2004; Heikkinen et al., 2002; Markham et al., 2002; Frick et al., 2002; Galea et al., 2002; Holmes et al., 2002; Korol and Kolo, 2002; Gureviciene et al., 2003). Although the factors underlying these inconsistencies are unknown, the inconsistencies are likely to be attributable, in large part, to the fact that the learning tasks examined differ a great deal in terms of both hippocampal involvement and memory demand. However, the fact that the hormone replacement regimens employed in these studies vary greatly suggests that they are also likely to be related to differences in the dose, duration, timing, and pattern of hormone administration and, moreover, whether progesterone is given in conjunction with estrogen. Although some of the regimens are more naturally relevant than others, there does not appear to be a clear correlation between natural relevance and effectiveness of estrogen in cognitive enhancement. Thus, precise replication of the natural ovarian cycle does not appear to be a requirement for overall cognitive enhancement.

At present, a role of adult-generated neurons in estrogen-associated enhancements of hippocampal-associated function has not been investigated. Although

multiple mechanisms are likely to participate in mediating the cognitive effects of estrogen (Woolley, 1999), evidence indicating that new cells may be important for some types of learning and memory (Gould et al., 1999; Shors et al., 2001, 2002) raises the possibility that increases in neuron production are involved. To evaluate this possibility, it is necessary to take into consideration the fact that new cells require time to become incorporated into existing circuitry in order to exert a functional impact. Previous work has indicated that new cells extend axons into anatomically appropriate regions as early as 4–10 days following BrdU incorporation (Hastings and Gould, 1999). Thus, for estrogen-induced enhancements of cognition to be attributable to an alteration in neuron production, the onset of hormone replacement would have to occur at least 4–10 days prior to the learning period. The majority of studies that have examined the impact of hormone replacement under such temporal conditions generally indicate a beneficial impact of estrogen treatment (Luine et al., 1998; Fader et al., 1998, 1999; Vongher and Frye, 1999; Gibbs, 2000; Heikkinen et al., 2002; Markham et al., 2002; Galea et al., 2002; Rissman et al., 2002; Gureviciene et al., 2003). Moreover, the cognitive enhancements observed during this time frame appear to be associated with low to moderate, but not high, levels of estrogen replacement (Bimonte and Denenberg, 1999; Galea et al., 2002).

Collectively, the results of studies examining estrogen effects on hippocampal cognition within a relevant time frame are consistent with a possible involvement of newly generated neurons. However, it is important to bear in mind that the multiple mechanisms through which estrogen exerts its influence on this brain region are likely to be somewhat overlapping, making it difficult to determine what role, if any, new neurons play. A better understanding of estrogen's regulation of neuron production, as well as its influence on hippocampal-dependent learning, will be required to establish the existence of a relationship between these two phenomena. If it is indeed the case that these two processes are related, the observation that OVX chronically decreases neuron production may have important implications for understanding the functional consequences of menopause and the replacement regimens often used to address it. Moreover, our findings that standard ovarian hormone replacement regimens do not restore normal patterns of cell proliferation suggest that potential cognitive benefits attributable to estrogen-induced changes in neuron production require a schedule that bears greater resemblance to one characteristic of normal physiology than those currently being employed.

Methodological note regarding p-histone H3 as a marker of hippocampal cell proliferation

The results of the present study indicate that expression of the endogenous M-phase marker p-histone H3 may be used as a reliable assay of hippocampal cell proliferation. p-Histone H3-labeled cells exhibited the morphology of cells undergoing mitosis and a distribution throughout the dentate gyrus similar to that of BrdU-labeled cells in animals surviving 2 hours post-BrdU injection. Although the within-animal correlation between the numbers of proliferating cells as determined by the p-histone H3 and BrdU methodologies was significant only in experiment 1, the data regarding the effect of various hormone manipulations yielded by the two markers were identical in all experiments in which both markers were employed. The reasons for the lack of a within-animal correlation in the latter experiments are not clear. However, some possible explanations are that 1) the sensitivity of the p-histone H3 assay is not sufficient to detect individual differences across animals within a statistical population, 2) the variability associated with the BrdU and the p-histone H3 methodologies prevent a correlation from being identified with the sampling methods that were used in the present study, or 3) small individual differences exist in the relative lengths of the S and M phases.

The use of p-histone H3 as a proliferation marker has several advantages. Because it is an endogenous protein, its expression is not influenced by differences in blood circulation, metabolism, or blood–brain barrier permeability. Although not a matter of concern when comparisons are being made across all experimental conditions that do not alter these aspects of physiology, consideration of these issues becomes critical under conditions such as seizure (Carpentier et al., 1990); exercise (Delp et al., 2001); stress (Endo et al., 1997, 1999); or, as in this study, hormone manipulation (Ziylan et al., 1990; Oztas and Kaya, 1998). Additionally, the fact that immunolabeled cells exhibit distinct morphological characteristics corresponding to the different phases of mitosis allows for an independent means of confirmation that 1) immunolabeled cells are indeed undergoing mitosis and 2) all of the cells in mitosis are being considered in the quantitative analysis.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Stephen Alves and Tracy Rydel for their valuable feedback during the development of these studies.

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