Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats.

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Abstract

Estradiol has been shown to have neuroprotective effects, and acute estradiol treatment enhances hippocampal neurogenesis in the female brain. However, little is known about the effects of repeated administration of estradiol on the female brain, or about the effects of estradiol on the male brain. Gonadectomized male and female adult rats were injected with BrdU (200 mg/kg), and then 24 h later were given a s.c. injection of either estradiol benzoate (33 µg/kg) or vehicle daily for 15 days. On day 16, animals were perfused and the brains processed to examine cells expressing Ki-67 (cell proliferation), BrdU (cell survival), doublecortin (young neuron production), pyknotic morphology (cell death), activated caspase-3 (apoptosis), and Fluoro-Jade B (degenerating neurons) in the dentate gyrus. In female rats, repeated administration of estradiol decreased the survival of new neurons (independent of any effects on initial cell proliferation), slightly increased cell proliferation, and decreased overall cell death in the dentate gyrus. In male rats, repeated administration of estradiol had no significant effect on neurogenesis or cell death. We therefore demonstrate a clear sex difference in the response to estradiol of hippocampal neurogenesis and apoptosis in adult rats, with adult females being more responsive to the effects of estradiol than males.

Keywords: sex differences; cell survival; hippocampal neurogenesis; dentate gyrus; estrogen

Abbreviations:

BrdU: 5-bromo-2-deoxyuridine

DG: dentate gyrus

ER: estrogen receptor

FJ: Fluoro-Jade B

GCL: granule cell layer

PBS: phosphate-buffered saline

SGZ: subgranular zone

Estradiol has been shown to have multifaceted neuroprotective effects: estradiol enhances neurotrophic factors (Gibbs, 1999, Scharfman et al., 2005), enhances hippocampal neurogenesis (Ormerod et al., 2003b), and suppresses cell death (Wise et al., 2001). There are sex differences in the efficacy with which estradiol protects neurons (Murray et al., 2003, Heyer et al., 2005), suggesting that sex can play a significant role in expression of hormonal neuroprotection.

Neurogenesis continues throughout life in specific regions of the mammalian brain, including the dentate gyrus of the hippocampus. Neurogenesis includes the related processes of cell proliferation (production of new cells) and cell survival (cells surviving to maturity). In adult rodents, there are sex differences in the amount of hippocampal cell proliferation and cell survival (Galea and McEwen, 1999, Westenbroek et al., 2004), and in the effects of reproductive status and stress on neurogenesis in the hippocampus (Galea and McEwen, 1999, Falconer and Galea, 2003, Westenbroek et al., 2004). For example, female meadow voles show seasonal changes in hippocampal cell proliferation, but no such changes exist in male meadow voles (Galea and McEwen, 1999). In addition, the effects of both acute and chronic stress on hippocampal neurogenesis differ between male and female rats (Falconer and Galea, 2003, Westenbroek et al., 2004). Although one study failed to find evidence for a significant sex difference in basal rates of hippocampal cell proliferation in mice, the direction of the means favored females (Lagace et al., 2007) and they did not examine females in proestrus against males, which is the only time a sex difference is observed in laboratory rats (Tanapat et al., 1999). Generally, when sex

differences exist, gonadal hormones regulate these differences. Although most of the literature to date has focused on the effects of estradiol on cell proliferation in *female* rodents (Tanapat et al., 1999, Ormerod and Galea, 2001, Ormerod et al., 2003a, Ormerod et al., 2003b, Tanapat et al., 2005), estradiol can also affect the male brain (Nelson and Bulun, 2001, Ormerod et al., 2004, Veiga et al., 2005). Thus, in the present experiment we examined the influence of estradiol on adult hippocampal neurogenesis in both male and female rats.

Acute estradiol enhances cell proliferation and decreases cell death in the dentate gyrus of adult female rats (Tanapat et al., 1999, Banasr et al., 2001, Ormerod et al., 2003b). Fewer studies have examined the effects of repeated or chronic estradiol treatment on hippocampal neurogenesis. Chronic estradiol administration does not affect cell proliferation in the dentate gyrus of adult female rats (Tanapat et al., 2005). However, estradiol administration doubles the number of new neurons in the dentate gyrus of male meadow voles, although only when estradiol is administered throughout the axon extension phase of these new neurons (Ormerod et al., 2004). The effects of repeated or chronic administration in particular are important to establish because clinical applications of estradiol, including estrogen replacement therapy for postmenopausal women, typically involve long-term administration rather than a single, acute dose. To date, no studies have examined the effects of repeated administration of estradiol on cell survival in adult female rodents. Furthermore, there have been very few studies directly comparing neurogenesis between the sexes (Galea and McEwen, 1999, Tanapat et al., 1999, Lagace et al., 2007) and no studies examining sex differences in the effects of

estradiol on neurogenesis in the hippocampus. If we are to use our knowledge of how adult neurogenesis is regulated to develop strategies to repair neuron loss in neurodegenerative diseases, it seems reasonable that we should also test neurogenic regulatory factors, such as gonadal hormones, in both male and female rats. Cell survival can be measured directly, as the survival of cells labeled during cell division, or indirectly, using markers of cell death. The former measure allows for determination of a specific timeline in the life cycle of a relatively new cell, whereas the latter allows for a measure of overall cell death, including both new neurons and older cells. In the present study, we examined the effects of repeated administration of estradiol on cell proliferation, survival of new cells, and cell death in the dentate gyrus of gonadectomized adult male and female rats. Cell proliferation was quantified as the number of cells containing Ki-67, an endogenous protein present in actively cycling cells (Kee et al., 2002, Schmidt et al., 2003, Zacchetti et al., 2003). Cell survival was quantified as the number of cells containing bromodeoxyuridine (BrdU), a thymidine analog that incorporates itself into the DNA of cells during the synthesis phase of the cell cycle. The survival of young neurons was further quantified as the number of cells with processes containing doublecortin (DCX), an endogenous microtubule-associated protein present in immature neurons (Gleeson et al., 1999, Rao and Shetty, 2004). Cell death was measured by counting pyknotic (dying) cells, counting cells expressing activated caspase-3, an effector caspase that is activated in cells in the early stages of apoptosis (Porter and Jänicke, 1999), or by counting the number of cells immunoreactive with Fluoro-Jade B, an anionic fluorescein derivative that labels degenerating neurons (Schmued et al., 1997, Schmued and Hopkins, 2000). We chose repeated administration of estradiol as, to date,

no studies have investigated the effects of repeated estradiol administration on cell survival or cell death in male and female rodents. We chose to give 15 d of exposure as 15 days is commensurate with the time when the majority of new cells are beginning to express mature neuronal markers (Ormerod et al., 2004, Cameron et al., 1993). We expected that estradiol would decrease cell death and alter neurogenesis (cell survival and cell proliferation), and that there would be sex differences in these effects.

Experimental Procedures

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines regarding appropriate treatment of animals and were approved by the University of British Columbia. Every effort was made to minimize the number of animals used per group and to minimize the suffering of animals used throughout all experimental procedures.

Experimental Subjects

Ten male and ten female adult (80-90 days old) Sprague-Dawley rats (Charles River Canada, Québec, Canada) were kept on a 12-hour light-dark cycle (lights on at 0700), singly housed in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and Purina rat chow and tap water ad libitum.

Procedure

All subjects (n = 5 per group) were gonadectomized under halothane anaesthesia one week after their arrival in the colony. Briefly, all rats were anaesthetized using 2-

bromo-2-chloro-1,1,1,-trifluoroethane (halothane, MTC Pharmaceuticals, Cambridge, Canada) using an initial flow rate of 4% and a maintenance flow rate of 2% during surgery. All females were bilaterally ovariectomized through bilateral flank incisions, and all males were bilaterally castrated through an incision in the scrotal sac. Eight days after surgery (Day 0), all rats received an i.p. injection of BrdU (200 mg / kg: Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in 0.9% saline. Injecting rats with BrdU prior to estradiol administration allows the examination of the effects of estradiol on the survival of new cells independently of its effects on cell proliferation. Beginning 24 hours after BrdU injections (Day 1), rats received s.c. injections of either estradiol benzoate (EB, dissolved in 0.1 mL sesame oil) or sesame oil (vehicle, 0.1 mL) each day for 15 consecutive days. The male rats used were approximately 1.5X the size of the females (mean male body mass: 450 g; mean female body mass: 282 g), so to maintain similar EB dose per unit body mass, and thus present the brain with a similar plasma concentration of EB, females were given 10 µg EB per injection, while males were given 15 µg EB per injection. All animals were injected between 1000 h and 1230 h each day. A dose of 33 µg/kg EB was used to match previous studies investigating the effects of estradiol on hippocampal neurogenesis, spine density and sexual behaviour in the female rodents (e.g. Woolley and McEwen, 1993, Tanapat et al., 1999, Rössler et al., 2006). Castrated male rats have recently been given a similar dose of EB as adults (Spritzer and Galea, 2007).

Tissue processing

On the day following the last injection (Day 16), rats were anaesthetized using an overdose of sodium pentobarbital (Euthanyl, MTC Pharmaceuticals, Cambridge, ON),

and perfused via cardiac puncture with 0.9% saline followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed and post-fixed for 24 hours in 4% formaldehyde in phosphate buffer, and stored in 0.1 M phosphate-buffered saline (PBS, pH 7.4) until sectioning. Brains were sectioned into 40 μ m coronal slices using a vibrating microtome VT1000S (Leica) and stored in 0.2% sodium azide in PBS until staining. For Ki-67, BrdU, cresyl violet and activated caspase-3 staining procedures, every tenth section through the entire hippocampus was stained and counted; for DCX and Fluoro-Jade staining, every twentieth section was used. For all staining with fluorescent antibodies, stained sections were mounted on plain glass microscope slides and coverslipped using PVA-DABCO.

Cell proliferation was quantified as the number of cells containing Ki-67 (Ki-67+ cells), an endogenous protein present in actively cycling cells (Fig. 2a). New cell survival was quantified as the number of cells containing BrdU (BrdU+ cells) 16 days after BrdU administration (Fig. 2b); BrdU was injected prior to estradiol administration, to focus on the influence of estradiol on survival independent of its well-known effects on cell proliferation. Young neuron production was quantified as the number of cells containing DCX (DCX+ cells), an endogenous protein present in migrating neurons (Fig. 2c). Cell death was measured by counting pyknotic cells (Fig. 2d), and by counting cells expressing activated caspase-3 (ActC3+ cells), an effector caspase that is activated in cells in the early stages of apoptosis (Fig. 2e), or immunoreactive with Fluoro-Jade B dye, which stains degenerating neurons (Fig. 2f).

Measures of cell death

Pyknotic cells were visualized with cresyl violet staining. For cells containing activated caspase-3, the selected sections were rinsed three times in 0.1 M PBS for 10 minutes each, then incubated with rabbit anti-activated-caspase-3 IgG primary antibody (AbCam, Cambridge, MA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. Sections were again rinsed three times in PBS, then incubated with donkey anti-rabbit FITC-conjugated IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 4 hours.

To observe degenerating neurons, tissue was rinsed three times in PBS, then mounted on slides and left to air-dry for 48 hours at room temperature. The slides were soaked in 0.06% potassium permanganate for 15 minutes, followed by soaking for 2 minutes in deionized water. The slides were then soaked in 0.0004% Fluoro-Jade solution (Chemicon, Temecula, CA) in 0.1% acetic acid, rinsed three times in deionized water, dried rapidly, cleared in xylene, and coverslipped with Permount (Fisher Scientific Canada, Ottawa, ON).

Measures of cell proliferation and new cell survival

To observe cells containing Ki-67, sections were rinsed three times in PBS, then incubated with mouse anti-Ki-67 IgG primary antibody (Vector Laboratories, Burlington, ON) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. The sections were again rinsed three times in PBS, then incubated in donkey anti-mouse Cy3-conjugated IgG

secondary antibody (Jackson ImmunoResearch, West Grove, PA) in 0.3% Triton X-100 in PBC (1:200) at 4°C for 2 hours.

To observe cells containing BrdU (new cell survival), sections were rinsed three times in PBS, then incubated in 2 N hydrochloric acid for 30 minutes at 37°C and rinsed three times in PBS. The tissue was incubated with 3% normal donkey serum in 0.3% Triton X-100 in PBS for 30 minutes, then with mouse anti-BrdU IgG primary antibody (Roche, Laval, QC) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. The sections were again rinsed three times in PBS, then incubated with donkey anti-mouse Cy3-conjugated IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 10 hours.

To observe cells containing DCX (young neuron production), sections were rinsed three times in PBS, then incubated in 0.05% Triton X-100 in sodium citrate buffer (pH 6.0) for 30 minutes at 85°C. The tissue was allowed to cool to room temperature, then incubated with 3% normal donkey serum in 0.3% Triton X-100 in PBS for 30 minutes, then with rabbit anti-DCX IgG primary antibody (Cell Signaling Technologies, Danvers, MA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 18 hours. The sections were again rinsed three times in PBS, then incubated with donkey anti-rabbit Alexa488-conjugated IgG secondary antibody (Invitrogen Canada, Burlington, ON) in 0.3% Triton X-100 in PBS (1:200) at room temperature for 18 hours.

Cell counting

All slides were coded prior to analysis so the experimenter was blind to treatment condition and sex. Cells containing Ki-67 (Ki-67+), BrdU (BrdU+), or active caspase-3 (active caspase-3+), or having a pyknotic morphology were counted throughout the granule cell layer, including the subgranular zone (SGZ, defined as an area 50 μm wide adjacent to the inner edge of the granule cell layer, e.g. Palmer et al., 2000) and hilus, provided their entire nucleus was visible (Fig. 1). Cells with long processes and containing DCX (DCX+) or cells that were immunoreactive with Fluoro-Jade B dye (FJ+) were counted throughout the granule cell layer, including the SGZ but not the hilus. All counting was done using a fluorescent E600 microscope (Nikon) either under brightfield (pyknotic cells) or fluorescent illumination (Ki-67+, BrdU+, DCX+, active caspase-3+, or FJ+ cells). Stereological estimates of the total number of cells in the dentate gyrus were generated by the exclusion of cells touching the uppermost focal plane of each slice and multiplying the total counts by 10 (Gundersen et al., 1988, West et al., 1991).

The area of the granule cell layer (including the SGZ) and hilus was measured in cresyl-stained sections using the digitizing software Act-1 (Nikon), and the volume for each area was calculated separately using Cavalieri's principle (Gundersen et al., 1988). Because there were differences between groups in dentate gyrus volume (Table 1), density scores for all cell counts were generated by taking the stereological estimates of cell number in either the granule cell layer (GCL) or hilus and then dividing this by the total volume of the respective region (GCL or hilus) to generate cell counts per mm^3 .

Phenotyping of cells containing BrdU

To determine the phenotype of cells containing BrdU, sections were rinsed three times in PBS. The tissue was blocked in 0.1 M PBS/1% NDS/3% Triton X-100 for 30 min at room temperature, then incubated with mouse anti-NeuN (1:200, Chemicon, Temecula, CA) primary antibody in 0.3% Triton X-100 in PBS at 4°C for 48 hours. The tissue was rinsed in PBS, and then incubated with fluorescent donkey anti-mouse Alexa 488 (1:200, Invitrogen, Burlington, ON) secondary antibody for 4 hrs at room temperature in the dark; the stained tissue was kept in the dark for the remainder of the staining procedure. The tissue was rinsed three times in PBS, incubated in 4% formaldehyde at room temperature for 10 min, then incubated in 2N HCl at 37°C for 30 min. The tissue was rinsed in PBS again and blocked in 0.1 M PBS/1% NDS/3% Triton X-100 for 30 min at room temperature, then incubated in rat anti-BrdU primary antibody (1:200, Oxford Biotechnology, Oxfordshire, UK) at 4°C for 10 hours. The tissue was rinsed in PBS, incubated in donkey anti-rat Cy3-conjugated secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) for 4 hrs at room temperature. Using a fluorescent microscope, 25 BrdU-labelled cells in each of 3 animals per treatment group were scored for co-expression with NeuN, indicating a neuronal phenotype (Mullen et al., 1992). Cell phenotype was verified on a subset of BrdU-labelled cells with confocal microscopy (Zeiss, Fig. 2g).

Hormone assays

Blood was collected from the right atrium just prior to perfusion on day 16. Whole blood was allowed to clot at 4°C for 24 hours, and then spun in a rotating centrifuge (8000 rpm for 8 minutes). The serum was collected and stored at -20°C until

processing using a commercial colorimetric enzyme-linked immunosorbent assay (ELISA) kit (RDI, Flanders, NJ; cross-reactivity with other estrogens < 3%, intra-and inter-assay CV < 10%; analytical sensitivity 16 pg/mL). One millilitre of diethyl ether was added to 230 μ L of serum in borosilicate glass tubes to extract out the steroid hormones. The tubes were then centrifuged at 4°C (5 minutes at 1000 g) and frozen at -80°C for 20 minutes. The ether fraction was decanted, and then dried down under nitrogen at 37°C. The resulting crystalline hormones were re-dissolved in 75 μ L of stripped human serum and analyzed using the ELISA kit.

Data analyses

The densities of pyknotic cells and activated caspase-3+, BrdU+ and Ki-67+ cells were each analyzed using repeated-measures ANOVA, with sex (male, female) and treatment (oil, estradiol) as between-subjects factors, and area (granule cell layer, hilus) as the within-subjects factor. DCX+ and FJ+ cells were only counted in the GCL, so the densities of these cells were analyzed using two-way ANOVA, with sex and treatment as between-subjects factors. Data were further analyzed using the Newman-Keuls post-hoc test, or *a priori* tests subject to a Bonferroni correction. Spearman rank order correlations were used to correlate serum estradiol levels with histological measures for both treatment groups within each sex.

Results

Male rats have larger dentate gyrus volumes than female rats

Overall, as expected, the volumes of the granule cell layer and hilus of the hippocampus were larger in males than in females, and the hilus was larger than the granule cell layer, but there were no other significant effects (main effect of sex: $F_{1,16} = 15.6$, $p = 0.001$; main effect of area: $F_{1,16} = 819$, $p < 0.001$; all other effects except for sex x area: $p > 0.40$; Table 1). There was a significant sex x area interaction effect in volume ($F_{1,16} = 11.0$, $p = 0.004$), post-hoc tests revealed that males had larger hilar volume ($p < 0.001$) than females and a trend for a larger granule cell layer than females ($p = 0.058$; Table 1). In addition, total dentate gyrus (DG) volume, as determined by adding together the GCL and hilus volume for each animal was calculated and analyzed with an ANOVA. DG volume was larger in males than in females ($F_{1,16} = 15.8$, $p = 0.001$) but there was no significant main effect of estradiol treatment, and no significant sex x treatment interaction effect (all p 's > 0.40). To account for the sex differences in dentate gyrus volumes, cell counts were analyzed as total counts or as densities (total cells per unit volume, with cells in the GCL and SGZ divided by GCL+SGZ volume, and cells in the hilus divided by hilus volume).

Estradiol increases the density of proliferating cells in the granule cell layer of female, but not male, rats

There was a significant main effect of area ($F_{1,16} = 266$; $p < 0.001$), indicating that there was a higher density of Ki-67+ cells in the granule cell layer than in the hilus (see Table 3 for counts of cells in the hilus). There were no other significant main or interaction effects on the density of Ki-67+ cells in the dentate gyrus ($p \geq 0.17$). Although the ANOVA revealed no significant sex x treatment interaction effect ($F_{1,16} =$

1.08; $p = 0.31$), *a priori* tests indicated that estradiol treatment increased the density of Ki-67+ cells in the granule cell layer of female ($p = 0.027$) but not male rats ($p = 0.73$; Fig. 3). An ANOVA on the total number of Ki-67+ cells found similar results to those found with density; there was a higher number of Ki-67+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1,16} = 230$; $p < 0.001$), but no other significant main or interaction effects on the number of Ki-67+ cells in the dentate gyrus ($p > 0.23$). Within each sex, the effects of estradiol treatment on the total number of Ki-67+ cells were in the same direction as the effects on the density of Ki-67+ cells in the granule cell layer, but the *a priori* tests revealed a slight trend for estradiol treatment to increase Ki-67+ cells in females (Table 2; males: $p = 0.62$; females: $p = 0.07$).

Estradiol decreases the survival of BrdU+ cells in the granule cell layer of female, but not male, rats

There was a significant main effect of area ($F_{1,16} = 200$; $p < 0.001$), indicating that there was a higher density of BrdU+ cells in the granule cell layer than in the hilus (see Table 3 for counts of cells in the hilus). There were no other significant main or interaction effects on the density of BrdU+ cells in the dentate gyrus (region x treatment interaction effect: $F_{1,16} = 4.27$, $p = 0.06$; all other effects $p > 0.16$). Although the ANOVA revealed no significant sex x treatment interaction effect ($F_{1,16} = 2.12$; $p = 0.16$), *a priori* tests indicated that estradiol treatment decreased the density of BrdU+ cells in the granule cell layer of female ($p = 0.005$) but not male rats ($p = 0.80$; Fig. 4a). An ANOVA on the total number of BrdU+ cells in the dentate gyrus found similar results to those found with density; there was a higher number of BrdU+ cells in the granule cell

layer than in the hilus (main effect of area: $F_{1,15} = 105$; $p < 0.001$). There was also an area x treatment interaction effect ($F_{1,16} = 6.76$; $p = 0.02$), which *post hoc* testing revealed to be due to an estradiol-induced decrease in the number of BrdU+ cells in the granule cell layer ($p = 0.003$) but not in the hilus ($p = 0.66$). There were no other significant main or interaction effects on the number of BrdU+ cells in the dentate gyrus ($p \geq 0.20$). Within each sex, *a priori* tests revealed that estradiol significantly decreased the number of BrdU+ cells in the granule cell layer of females ($p = 0.009$) but not that of males ($p = 0.18$; Table 2).

There were no significant main or interaction effects on the density of DCX+ cells in the granule cell layer (all effects $p > 0.23$). *A priori* tests did not indicate any effect of estradiol treatment on the density of DCX+ cells in the granule cell layer of female ($p = 0.22$) or male rats ($p = 0.61$), although the direction of the means may suggest effects similar to those found in BrdU+ cells (Fig. 4c). An ANOVA on the total number of DCX+ cells in the granule cell layer found similar results to those found with density (Table 2; ANOVA: all effects $p > 0.12$; *a priori* comparison within females: $p = 0.24$, *a priori* comparison within males: $p = 0.29$).

Estradiol decreases cell death in the dentate gyrus of female but not male rats

Overall, females also had lower densities of pyknotic cells than males, estradiol decreased the density of pyknotic cells (Fig. 5a), and the density of pyknotic cells was higher in the granule cell layer than the hilus (main effect of sex: $F_{1,16} = 7.4$; $p = 0.01$; main effect of treatment: $F_{1,16} = 4.4$; $p = 0.05$; main effect of area: $F_{1,16} = 61$; $p < 0.001$;

see Table 3 for counts of cells in the hilus). In addition, the sex x treatment effect approached significance ($p = 0.08$), and from Figure 5 it is apparent that the main effect of estradiol reducing the density of pyknotic cells is driven by the data from the females. *A priori* tests confirmed that estradiol reduced the density of pyknotic cells in the granule cell layer of female ($p = 0.02$) but not male rats ($p = 0.60$; Fig. 5a). An ANOVA on the total number of pyknotic cells in the dentate gyrus found similar results as those found with density; females had fewer pyknotic cells than males, estradiol decreased the number of pyknotic cells, and there were more pyknotic cells in the granule cell layer than in the hilus (main effect of sex: $F_{1,16} = 14.6$; $p = 0.001$; main effect of treatment: $F_{1,16} = 6.33$; $p = 0.02$; main effect of area: $F_{1,16} = 9.39$; $p = 0.007$), and no significant interaction effects ($p > 0.14$). Within each sex, *a priori* tests revealed that estradiol decreased the number of pyknotic cells in the granule cell layer of females ($p = 0.03$) but not that of males ($p = 0.23$; Table 2).

There was a higher density of ActC3+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1,16} = 151$; $p < 0.001$; see Table 3 for counts of cells in the hilus), but no other main or interaction effects on the density of ActC3+ cells in the granule cell layer ($p \geq 0.14$). Although the ANOVA revealed no significant sex x treatment interaction effect ($F_{1,16} = 1.68$; $p = 0.21$), *a priori* tests revealed that estradiol treatment decreased the density of cells containing activated caspase-3 in the granule cell layer of female ($p = 0.02$) but not male rats ($p = 0.64$; Fig. 5b). An ANOVA on the total number of ActC3+ cells in the dentate gyrus found similar results as those found with density; there were more ActC3+ cells in the granule cell layer than in the hilus (main

effect of area: $F_{1,16} = 120$; $p < 0.001$), and no other significant main or interaction effects ($p > 0.25$). Within each sex, *a priori* tests revealed that estradiol decreased the number of ActC3+ cells in the granule cell layer of females ($p = 0.01$) but not that of males ($p = 0.83$; Table 2).

There was a trend for a sex x treatment interaction effect on the density of FJ+ cells in the granule cell layer ($F_{1,15} = 3.85$; $p = 0.068$), but no main effect of sex or treatment (main effect of sex: $F_{1,15} = 0.03$; $p = 0.87$; main effect of treatment: $F_{1,15} = 2.94$; $p = 0.11$). *A priori* tests revealed that estradiol treatment decreased the density of FJ+ cells in the granule cell layer of female ($p = 0.02$) but not male rats ($p = 0.87$; Fig. 5c). An ANOVA on total numbers of FJ+ cells found similar results as those found with density; there was a trend both for a main effect of treatment and for a sex x treatment interaction effect, but no significant main effect of sex on the total number of FJ+ cells (main effect of treatment: $F_{1,15} = 3.77$; $p = 0.07$; sex x treatment interaction effect: $F_{1,15} = 3.33$; $p = 0.09$; main effect of sex: $F_{1,15} = 1.25$; $p = 0.28$). Within each sex, *a priori* tests revealed that estradiol decreased the number of FJ+ cells in the granule cell layer of females ($p = 0.02$) but not that of males ($p = 0.94$; Table 2).

Phenotype of surviving BrdU+ cells

The percentage of cells co-expressing BrdU and NeuN (BrdU+/NeuN+) was approximately 81% in the granule cell layer in both males and females. There were no significant differences among groups in the proportion of BrdU+ cells in the granule cell layer that co-expressed NeuN ($0.56 < p < 0.95$; Table 4). When we multiplied the

proportion of BrdU+/NeuN+ cells by the total number of BrdU+ cells to estimate the total number of new neurons produced, we found that overall estradiol decreased both the density of new neurons produced (Fig. 4b; main effect of treatment: $F_{1,5} = 7.8$; $p = 0.04$) and the total number of new neurons produced (Table 2; $F_{1,5} = 10.6$; $p = 0.02$). Although there was no main effect of sex on new neuron density, and no other significant main or interaction effect on total new neuron numbers (all p 's > 0.19), there was a significant sex x treatment interaction effect on new neuron density ($F_{1,5} = 6.46$; $p = 0.05$). Post hoc tests revealed that the effect of treatment on the density of new neurons was significant in females ($p = 0.046$) but not in males ($p = 0.87$). In addition, *a priori* tests revealed that the effect of treatment on the total number of new neurons produced was significant in females ($p = 0.02$) but not in males ($p = 0.29$).

Serum estradiol levels

Estradiol levels in all oil-injected rats were below the detection threshold of the ELISA kit (8 pg/mL); the mean estradiol level of estradiol-injected females (60.1 ± 10.3 pg/mL), did not significantly differ from that of estradiol-injected males (73.7 ± 9.5 pg/mL; $p = 0.19$).

Correlations

In keeping with the above results showing no significant effect of estradiol on neurogenesis and cell death in the male hippocampus, there were no significant correlations between any of these measures and estradiol level in males (all p 's > 0.2). However, there were significant correlations between estradiol level and

neurogenesis/cell death measures in female rats (Fig. 6). The density of BrdU+ cells in the granule cell layer varied negatively with estradiol level in female rats ($r = -0.78$, $p = 0.007$), indicating that as estradiol level increased, fewer new cells survived; estradiol level accounted for approximately 61% of the variation in the density of BrdU+ cells in females. There was also a trend for a significant negative relationship between the total number of BrdU+ cells in the granule cell layer and serum estradiol level in female rats ($r = -0.60$, $p = 0.066$), suggesting that as estradiol levels increased there was a subsequent decrease in the number of BrdU+ cells surviving.

Similarly, estradiol level was negatively related in females with the number of pyknotic cells in the granule cell layer ($r = -0.63$, $p = 0.049$) and the hilus ($r = -0.79$, $p = 0.006$), as well as the density of pyknotic cells in the granule cell layer ($r = -0.64$, $p = 0.046$) and the hilus ($r = -0.85$, $p = 0.002$), indicating that as estradiol level increased, the number and density of pyknotic cells decreased; estradiol level thus accounted for over 40% of the variation in the number and density of pyknotic cells in females in the granule cell layer, and over 60% of the variation in the number and density of pyknotic cells in the hilus. Estradiol level was also negatively correlated with the number of FJ+ cells ($r = -0.66$, $p = 0.0003$), as well as the density of FJ+ cells in the granule cell layer ($r = -0.65$, $p = 0.0005$), indicating that as estradiol level increased, the number and density of FJ+ cells decreased; estradiol level thus accounted for over 40% of the variation in the number and density of FJ+ cells in the granule cell layer. The other measures of neurogenesis and cell death examined did not vary significantly with serum estradiol level (all $p \geq 0.33$), nor were any of the correlations between measures of neurogenesis or cell death statistically significant in males (all $p \geq 0.1$).

Discussion

The results from the present study demonstrate that estradiol regulates cell death and neurogenesis in the hippocampus of female, but not male rats. Repeated (15-day) administration of estradiol resulted in a decrease in cell death and new neuron survival, with a slight increase in cell proliferation by the end of treatment, in the dentate gyrus of adult female, but not male, rats. The finding that estradiol decreases overall cell death is consistent with previous reports demonstrating that estradiol can protect neurons against a variety of insults (e.g. Goodman et al., 1996, Garcia-Segura et al., 2001, Lee and McEwen, 2001, Galanopoulou et al., 2003). In light of this, the decrease in BrdU+ cell survival we found may seem paradoxical (decreasing BrdU+ cell survival while decreasing overall cell death), but it is important to note that the dying (pyknotic, activated caspase-3+, and FJ+) cells likely belong to a different population of cells than those that had previously been labelled with BrdU. The BrdU+ cells are likely older than the dying cells observed, because the majority of cells that die in the dentate gyrus do so at early maturation stages, rather than at later stages (Dayer et al., 2003).

Consistent with previous reports, although males had larger dentate gyrus volumes than females, repeated estradiol administration had no significant effect on dentate gyrus volume in either sex (Roof and Havens, 1992, Galea et al., 1999). However, it should be noted that early exposure to testosterone increases granule cell layer volume (Roof and Havens, 1992), and chronic high estradiol levels are correlated with larger total hippocampus volume in the female meadow vole (Galea et al., 1999). Long-term estradiol administration had no significant effect on cell proliferation,

survival, or death in adult gonadectomized male rats. This is consistent with findings that there is no fluctuation in cell proliferation across reproductive status (seasons) in the dentate gyrus of male meadow voles (Galea and McEwen, 1999), no difference in cell proliferation in the dentate gyrus of castrated adult male rats (Spritzer and Galea, 2007) nor changes in cell survival with estradiol in adult male rats (Spritzer and Galea, 2007). The finding that estradiol did not significantly affect new neuron survival in male rats is somewhat inconsistent with Ormerod et al. (2004), who found that 5 days of estradiol treatment enhanced new neuron survival in males, but only when administered during the period in which the new cells were likely extending their axons. Thus the length of exposure to estradiol, and when during the cell maturation cycle estradiol exposure occurs, may determine whether estradiol can affect hippocampal neurogenesis in male rodents.

Repeated estradiol slightly increased the density of proliferating cells after repeated exposure in female but not male rats

After 15 daily injections of estradiol administration, cell proliferation, as measured by the density (but not total number) of cells expressing Ki-67, was increased in the GCL of female, but not male rats. Acute administration of estradiol increases cell proliferation in the GCL after 4h, but suppresses cell proliferation 48h later in adult female rats (Ormerod et al., 2003b). Other studies have found that estradiol exposure for 3 days results in no significant effect on hippocampal cell proliferation (3 days; Perez-Martin et al., 2003, Fowler et al., 2005). In contrast to the present findings, repeated administration of estradiol to ovariectomized rats via implanted pellets for 3 weeks does

not appear to affect cell proliferation in the dentate gyrus of adult female rats (Tanapat et al., 2005). However, there are several differences between that study and our own. First, in the present study we administered estradiol via daily s.c. injections, rather than via pellets as used in the Tanapat et al. study; pulsatile exposure to estradiol via daily injections may have different effects than continuous exposure to a constant high serum concentration of estradiol. Although Tanapat et al. did include a treatment group receiving pulsatile estradiol injections and did not find an effect on cell proliferation, their estradiol injections were 4 days apart, compared to our 24-hr spacing of injections, which may contribute to the differences between their results and those obtained in the current study. Second, we used 17 β -estradiol benzoate whereas Tanapat et al. used unconjugated 17 β -estradiol, which has a shorter biological half-life and it is possible that the clearance rate of the particular type of estradiol used may affect the cellular response to estradiol. Third, we administered estradiol benzoate for 15 d whereas Tanapat et al. used implanted pellets for 21 days and over longer time periods the effects of estradiol may be attenuated as the system adjusts to the administration schedule. Fourth, we examined Ki-67, an endogenous protein that labels all actively cycling cells, instead of BrdU which was used by Tanapat et al. (2005) which only labels cells that are in the synthesis phase of the cell cycle within a 2 h period (Packard et al., 1973). BrdU may be insensitive to changes in proliferation if estradiol affects the length of stages of the cell cycle other than the S-phase. Furthermore, estradiol can alter blood-brain barrier permeability after long-term treatment (Ziylan et al., 1990, Bake and Sohrabji, 2004), thus it is entirely possible the results found by Tanapat et al., (2005), in terms of BrdU-labeled cells, were confounded by effects of estradiol on blood-brain barrier permeability.

Because we used an endogenous marker of proliferation (Ki-67) our results would not be influenced by blood-brain barrier permeability changes as a result of repeated estradiol. However, this explanation does not account for the fact that Tanapat et al. (2005) found the same result, namely no significant difference in cell proliferation after 21 days of estradiol treatment using the endogenous marker p-histone H3. Interestingly, in this part of their study they found that the number of p-histone H3-labeled cells did not correlate with the number of BrdU-labeled cells. Thus the difference between our findings with Ki-67 and their findings with p-histone H3 may be due to the fact that Ki-67 is present in cycling cells for a longer period of time than p-histone H3. Finally, we analyzed both total cell numbers and cell densities, and found a statistically significant difference only in the latter measure, whereas Tanapat et al. only analyzed total cell numbers.

Repeated estradiol suppresses new cell and new neuron survival in female but not male rats

Repeated exposure to estradiol *reduced* the number of surviving BrdU+ cells in female, but not male, rats. BrdU was administered prior to estradiol exposure, so our results demonstrate the effect of estradiol on survival of these labelled cells, rather than having any effect on their initial proliferation or BrdU incorporation rate. Short-term estradiol has been shown to increase cell survival in adult male meadow voles (Ormerod et al., 2004), and acute estradiol tends to protect neurons against a variety of insults (e.g. Garcia-Segura et al., 2001, Lee and McEwen, 2001, Galanopoulou et al., 2003), but few studies have investigated the effects of *repeated* estradiol on neuroprotection. Similarly, the reduction in the number of BrdU+ cells surviving in our study is consistent with

findings in female meadow voles that high levels of estradiol for 5 weeks results in lower numbers of ^3H -thymidine-labelled cells surviving compared to voles with low levels of estradiol (Ormerod and Galea, 2001). In addition, neither the sex of the animal nor estradiol treatment affected the percentage of BrdU+ cells that co-expressed NeuN, a protein found in mature neurons, consistent with previous reports that repeated estradiol administration does not necessarily regulate the differentiation of newly generated cells in the adult dentate gyrus (e.g. Tanapat et al., 1999, Perez-Martin et al., 2003, Ormerod et al., 2004). Thus, overall we found that repeated estradiol treatment decreased the survival of new neurons in female but not male rats.

Repeated estradiol suppressed cell death in female, but not male, rats

Repeated estradiol administration to female rats in the present study reduced the density of pyknotic, activated caspase-3+, and FJ+ cells, suggesting that cell death is suppressed in female rats with repeated exposure to estradiol. Ovariectomy itself appears to have increased the densities of activated caspase-3+ and FJ+ cells, which were reduced by estradiol administration in females. However, this same pattern was not seen when examining cells with a pyknotic morphology, as estradiol-injected females showed a reduction in cell death below that of all other groups, and indeed females overall had fewer pyknotic cells than males, in terms of both total numbers and density. A similar effect has been found in intact rats, where female rats in proestrus have fewer pyknotic cells in the dentate gyrus than males (Tanapat et al., 1999). Although counts of pyknotic cells and cells expressing activated caspase-3 can both be used to quantify cell death, these two markers target different time points in cell death, and perhaps slightly different

populations of cells. The precise nature of the degenerating neurons stained with Fluoro-Jade dyes is as of yet undetermined, so it is unknown whether it preferentially stains neurons at a particular stage of the degeneration process. However, it has been shown to overlap substantially with activated caspase-3 after neurotoxic insult (Scallet et al., 2004). Caspase-3 is activated early in the apoptotic cascade, whereas the pyknotic morphology presents later in the process of cell death (necrotic and apoptotic) and persists for up to 72 h before the cell is dismantled completely and is no longer visible (Hu et al., 1997). Thus it is possible that any effects of prolonged ovariectomy (as rats would have been deprived of ovarian hormones for 22 days at the time of perfusion) may be specific to the cell maturation stage(s) during which cells are deprived of ovarian hormones.

Repeated estradiol suppresses new cell survival and cell death: a paradox?

There are at several plausible explanations for the apparent discrepancy between the decreased in the number of dying cells (pyknotic, active caspase-3+, and FJ+) and the decrease in the number of BrdU+ surviving cells. First, the ‘dying’ cells observed in the present study may have been younger than the BrdU+ cells. New immature neurons are more likely to undergo cell death within the first week after birth (Cameron et al., 1993, Gould et al., 1999) and so the dying cells may have been exposed to estradiol for a shorter period of time than the BrdU+ cells that had been exposed to estradiol for a full 15 days. This is supported somewhat by the pattern of DCX+ cell densities, which roughly tracked the BrdU+ cell densities but do not reach statistical significance; most DCX+ cells are typically younger than 16 days (Brown et al., 2003), and as these cells mature in the presence of estradiol their survival may decrease. Duration of estradiol

exposure may strongly modulate estradiol's effects, as treatment of ovariectomized female rats with estradiol valerate for 12 days up-regulates the expression of estrogen receptor (ER)- β , but not ER α (Jin et al., 2005). Interestingly, progenitor cells in the hippocampus show higher expression of ER β than ER α in female rats (Mazzucco et al., 2006). Together these findings suggest that repeated exposure to estradiol may alter the responsiveness of cells to estradiol itself, potentially reversing the survival-enhancing effects of acute estradiol exposure.

Secondly, the dying cells observed in the present study may have been from an *older* population than the BrdU+ cells, and may have been rescued from death by estradiol administration. The preservation of older cells may have reduced the survival of younger cells as production of new neurons in the olfactory epithelium is inhibited by GDF11, a growth and differentiation factor that is produced by mature olfactory receptor neurons and/or their progenitors (Wu et al., 2003). Thus it is possible that a similar system operates in the granule cell layer of the hippocampus, whereby the presence of older neurons inhibits the production and/or maturation of additional, younger neurons. Alternatively, as repeated estradiol treatment enhances the excitatory effects of γ -aminobutyric acid (GABA) on young neurons (Nuñez et al., 2005), the addition of estradiol may cause GABA transmission to have excitotoxic effects on these cells (Nuñez and McCarthy, 2003). The BrdU+ cells we observed were up to 16 days old, within the 2- to 3-week-long early cell development stage during which GABA acts to depolarize immature neurons (Karten et al., 2006). Thus it is possible that estradiol enhances GABA-induced activation of immature cells, independently of its effects on both progenitor cells that do not yet received synaptic inputs and older neurons in which the

effect of GABA is inhibitory. Estradiol may specifically render young neurons susceptible to excitotoxic effects of GABA, thus decreasing the survival of BrdU+ cells in the present study, while having different (potentially neuroprotective) effects on both progenitor (Ki-67+) cells and mature neurons.

Finally, it is possible that the increased cell proliferation found in the GCL with repeated estradiol exposure caused a reduction of the BrdU concentration in surviving cells to below detectable limits. Thus, rather than indicating decreased survival, the apparent reduction in the number of BrdU+ cells in female rats may be due to increased proliferation diluting the BrdU signal. Our current data examining young neuron production unfortunately neither conclusively supports nor necessarily refutes this conclusion. Staining for the endogenous protein DCX revealed fewer DCX+ cells in females given estradiol, but the effect was not statistically significant, and further investigation into this possibility is warranted.

Sex differences in response to estradiol

Although repeated administration of estradiol had multiple effects on the cells in the dentate gyrus of female rats, we found no significant effects of repeated administration of estradiol in male rats. Although testosterone can be metabolized to estradiol, it has been shown to have neuroprotective properties independent of estradiol pathways (Freeman et al., 1996, Frye and McCormick, 2000). Chronic testosterone exposure promotes hippocampal neurogenesis via cell survival in males and this effect is mediated by dihydrotestosterone but not estradiol (Spritzer and Galea, 2007). Our findings are also consistent with studies in the nigrostriatal dopaminergic systems as

estradiol protects the nigrostriatal dopaminergic system against methamphetamine neurotoxicity in female mice, but not in male mice (Anderson et al., 2005). Coupled with our findings, these results suggest the female brain is more responsive to the effects of estradiol than the male brain, whereas the male brain may be more reliant on testosterone.

Interestingly, the normal amount of estradiol present in the brain may not differ much between the sexes; while precise concentrations are not yet known, male rats normally have a substantial amount of estradiol available in the brain, produced locally by the aromatization of testosterone (Naftolin et al., 1975); as aromatase activity is higher in male than female rats, it is conceivable that the local estradiol concentrations are similar (Wagner and Morrell, 1997). Thus it is unlikely that the sex differences in response to exogenous estradiol are the result of the same dose providing supra-physiological concentrations in one sex but not the other. There is no sex difference in the levels or regional localization of estrogen receptor (ER) subtypes α and β in the hippocampus of adult rats (Weiland et al., 1997, Kalita et al., 2005), so it is unlikely that any sex difference in response to estradiol is a result of differences in the regional distribution of estrogen receptors within the hippocampus. However, there may be differences in the types of cells on which estrogen receptors are located. For example, in intact female rats, young neurons in the dentate gyrus contain ER β (Herrick et al., 2006), and in ovariectomized female rats, cells expressing Ki-67 in the dentate gyrus contain mRNA for both ER α and ER β , though more Ki-67+ cells contain ER β than contain ER α , and only a small fraction (3-10%) of Ki-67+ cells co-localize with either estrogen receptor subtype (Mazzucco et al., 2006). In intact male rats, on the other hand, the majority of Ki-67+ cells (75-80%) contain ER α or β , and the two receptor subtypes do

not appear to differ in the extent to which they co-localize with Ki-67 (Isgor and Watson, 2005). These findings may seem paradoxical in light of our results demonstrating that estradiol affects cell proliferation in female but not male rats, as this is opposite to what would be expected if the effects of estradiol on proliferating cells depend on those cells expressing ERs. However, whereas Mazzucco et al. (2006) examined ovariectomized females, and the rats used in the current study were all gonadectomized, Isgor and Watson (2005) used intact males. It is possible that gonadectomy affects the cellular localization of ERs; ovariectomy in mice decreases overall ER levels in the brain (Ehret and Buckenmaier, 1994), so the removal of gonadal hormones may selectively reduce the level of ER expression in progenitor cells. This reduction in ER level may be specific to ER α , as the number of cells containing ER β is not changed 2 weeks after ovariectomy in adult female rats (Blurton-Jones and Tuszynski, 2002).

Possible mechanisms of estradiol-induced changes in hippocampal neurogenesis and cell death

The presence or absence of ER α or ER β on progenitor or daughter cells does not preclude cells from responding to estradiol via the putative membrane ER (Toran-Allerand et al., 2002) or through another mechanism (independent of or dependent on ERs), such as neurotrophins (Lee and McEwen, 2001, Scharfman and Maclusky, 2005). Intriguingly, previous research in our laboratory has found that agonists of neither ER α nor ER β enhance cell proliferation to the same extent as estradiol alone (Mazzucco et al., 2006). Furthermore, the ER antagonist ICI 182, 780, did not completely eliminate the estradiol-induced enhancement of cell proliferation (Nagy et al., 2006), and

administration of ICI 182,170 alone slightly enhanced cell proliferation. This ER antagonist has been suggested to be act as a ligand for the putative membrane-bound ER (Zhao et al., 2006). Thus these lines of evidence suggest that estradiol may be working through another mechanism independent of its two known ERs to upregulate cell proliferation. In addition to the membrane-bound ER, another such mechanism could be the neurotrophin, brain-derived neurotrophic factor (BDNF) (Scharfman and Macluskay, 2005).

Estradiol can upregulate BDNF through pathways dependent or independent of its ER (Scharfman and Macluskay, 2005). BDNF and its receptor TrkB are expressed at high levels in the hippocampus, and estradiol upregulates BDNF mRNA and protein in the hippocampus (Gibbs, 1999, Zhou et al., 2005) and increases BDNF release from neurons in the dentate gyrus (Sato et al., 2007). Estrogen response elements have been located on the genes for growth factors, including BDNF, indicating that estrogens can activate BDNF gene transcription (see Scharfman and Macluskay, 2005 for review). Many neurons and astrocytes in a variety of brain regions coexpress both TrkB receptors and ER (McCarthy et al., 2002). Furthermore, many structural and electrophysiological changes due to estradiol are mediated through estradiol's interactions with BDNF (Murphy et al., 1998, Scharfman and Macluskay, 2005).

Multiple lines of evidence indicate BDNF promotes hippocampal neurogenesis: dietary restriction and enriched environments have been shown to increase both hippocampal neurogenesis and BDNF (Lee et al., 2000, Lee et al., 2002); BDNF^{+/-} mice, with reduced BDNF levels, have reduced hippocampal neurogenesis (Sairanen et al.,

2005); and BDNF infusions upregulate hippocampal neurogenesis (Scharfman et al., 2005). Furthermore, BDNF is required for adult hippocampal progenitors to differentiate into neurons in vitro (Bull and Bartlett, 2005) and BDNF levels also modulate apoptosis (Kalb, 2005). Therefore it is likely that there is a connection between BDNF, neurogenesis, apoptosis and estradiol, and studies are currently underway to elucidate this link.

Conclusion

We have reported here the first evidence of a sex difference *in vivo* in the effects of repeated administration of estradiol on hippocampal neurogenesis and cell death. Although repeated estradiol exposure increased cell proliferation and decreased cell death in female rats, it had no such effect in male rats. As estradiol has been presented as a potential therapeutic or preventative agent for neurodegenerative diseases, it is important to note that while both men and women suffer from such diseases, the efficacy of any potential treatment or preventative agent cannot be verified in one sex alone. Additionally, although long-term estradiol decreased overall cell death in the female hippocampus, it also reduced the survival of cells that had been labelled with BrdU prior to estradiol treatment. As preventative or therapeutic medical treatments that include estrogens can be expected to consist of repeated or chronic administration, it is important to understand the effect of extended treatment on the brain. The effects of long-term estradiol administration differ from, and may be more complex than, those of acute administration, so caution should be used when evaluating the results of acute or short-

term studies in terms of the potential utility of estrogens in preventing or treating neurodegenerative diseases.

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Table 1: Effects of sex and chronic estradiol treatment on dentate gyrus volume.

The granule cell layer (GCL) and hilus in females were generally smaller than in males, and the hilus was larger than the granule cell layer in all groups. Data are shown as group mean $\pm$ standard error of the mean. ^astatistically significantly different from the same area in males receiving the same treatment, $p < 0.05$; ^bstatistically significantly different from hilus volume, $p < 0.05$.

<u>Sex</u>	<u>Treatment</u>	<u>Total GCL volume (mm³)</u>	<u>Total hilus volume (mm³)</u>
Female	Oil	$3.80 \pm 0.23^{a, b}$	7.36 ± 0.34^a
	Estradiol benzoate	3.72 ± 0.29^b	7.33 ± 0.50^a
Male	Oil	4.79 ± 0.23^b	9.27 ± 0.26
	Estradiol benzoate	4.29 ± 0.20^b	8.84 ± 0.57

Table 2:

Total counts of pyknotic cells and Ki-67+, BrdU+, and ActC3+ cells were lower in the hilus than in the granule cell layer ($p < 0.01$). Data are shown as group mean $\pm$ standard error of the mean. * statistically significantly different from oil-injected controls ($p < 0.05$).

<u>Cell type</u>	<u>Sex</u>	<u>Treatment</u>	<u>Total number of cells in GCL</u>	<u>Total number of cells in hilus</u>
Ki-67+	Female	Oil	8603 $\pm$ 1017	146 $\pm$ 36
		Estradiol benzoate	11290 $\pm$ 1548	157 $\pm$ 44
	Male	Oil	11696 $\pm$ 1480	328 $\pm$ 63
		Estradiol benzoate	10993 $\pm$ 1472	234 $\pm$ 85
BrdU+	Female	Oil	2885 $\pm$ 355	939 $\pm$ 141
		Estradiol benzoate	* 2035 $\pm$ 406	1037 $\pm$ 150
	Male	Oil	2824 $\pm$ 240	1068 $\pm$ 154
		Estradiol benzoate	2416 $\pm$ 113	1210 $\pm$ 166
DCX+	Female	Oil	11377 $\pm$ 1693	n/a
		Estradiol benzoate	7855 $\pm$ 1735	n/a
	Male	Oil	11887 $\pm$ 2041	n/a
		Estradiol benzoate	8533 $\pm$ 2602	n/a
BrdU+/NeuN+	Female	Oil	2614 $\pm$ 344	n/a
		Estradiol benzoate	* 1218 $\pm$ 95	n/a

Pyknotic	Male	Oil	2247 ± 462	n/a
		Estradiol benzoate	1735 ± 331	n/a
	Female	Oil	120 ± 19	108 ± 22
		Estradiol benzoate	* 66 ± 8	* 38 ± 8
ActC3+	Male	Oil	180 ± 19	118 ± 23
		Estradiol benzoate	152 ± 25	116 ± 21
	Female	Oil	3192 ± 436	918 ± 278
		Estradiol benzoate	* 2206 ± 378	732 ± 190
FJ+	Male	Oil	2830 ± 248	830 ± 107
		Estradiol benzoate	2754 ± 671	708 ± 150
	Female	Oil	104.0 ± 16.0	n/a
		Estradiol benzoate	* 40.0 ± 15.5	n/a
	Male	Oil	92.0 ± 13.6	n/a
		Estradiol benzoate	90.0 ± 23.8	n/a

Table 3:

Densities of pyknotic cells and Ki-67+, BrdU+, or ActC3+ cells were much lower in the hilus than in the granule cell layer ($p < 0.05$; compare to Figures 2-4). Data are shown as group mean $\pm$ standard error of the mean.

<u>Cell type</u>	<u>Sex</u>	<u>Treatment</u>	<u>Density of cells in hilus (per mm³)</u>
Ki-67+	Female	Oil	20.1 $\pm$ 5.0
		Estradiol benzoate	21.0 $\pm$ 5.5
	Male	Oil	35.5 $\pm$ 6.7
		Estradiol benzoate	28.0 $\pm$ 10.7
BrdU+	Female	Oil	127.4 $\pm$ 16.9
		Estradiol benzoate	142.4 $\pm$ 19.8
	Male	Oil	116.1 $\pm$ 18.3
		Estradiol benzoate	142.0 $\pm$ 26.7
Pyknotic	Female	Oil	14.3 $\pm$ 2.4
		Estradiol benzoate	5.0 $\pm$ 0.8
	Male	Oil	12.5 $\pm$ 2.2
		Estradiol benzoate	13.7 $\pm$ 2.9
ActC3+	Female	Oil	123.8 $\pm$ 34.9
		Estradiol benzoate	101.8 $\pm$ 25.2
	Male	Oil	89.9 $\pm$ 12.3
		Estradiol benzoate	83.3 $\pm$ 19.8

Table 4: Neither sex nor chronic estradiol treatment affected the percentage of BrdU+ cells that were co-labelled with NeuN 16 days after BrdU injection ($p > 0.05$). Data are shown as group mean $\pm$ standard error of the mean.

<u>Sex</u>	<u>Treatment</u>	<u>Percentage of BrdU+ cells</u> <u>co-labelled with NeuN</u>
Female	Oil	87 ± 12
	Estradiol benzoate	76 ± 10
Male	Oil	83 ± 12
	Estradiol benzoate	79 ± 12

Figure legends

Figure 1: Bright-field photomicrograph showing the subfields of the dentate gyrus of the hippocampus. Cells were counted in the hilus and the granule cell layer (GCL), which included the subgranular zone (SGZ). Cells expressing Ki-67, BrdU, or activated caspase-3, or cells expressing a pyknotic morphology, were counted throughout the GCL (including the SGZ) and the hilus.

Figure 2: Representative photomicrographs of cells in the dentate gyrus of adult rats after 15 days of estradiol administration. (A) To quantify cell proliferation, we counted cells containing Ki-67. To quantify young cell survival, we counted cells containing (B) BrdU or (C) DCX 16 days after BrdU injection. To quantify overall cell death, we counted cells (D) expressing a pyknotic morphology, (E) containing active caspase-3 or (F) that were immuno-reactive with a Fluoro-Jade stain. (G) Tissue was also double-labelled with fluorescent antibodies against BrdU (red) and NeuN (green) to determine whether BrdU-labelled cells were neurons.

Figure 3: Repeated estradiol benzoate administration increased cell proliferation in the granule cell layer of the dentate gyrus in adult female but not adult male rats, as indicated by the density of cells expressing Ki-67 per unit volume of the granule cell layer (including the subgranular zone). Bars show group mean + standard error of the mean. * statistically significantly different ($p < 0.05$) from oil-injected controls.

Figure 4: Repeated estradiol administration (A) decreased the survival of cells labelled with BrdU in the granule cell layer and subgranular zone of the dentate gyrus in adult female but not adult male rats and (B) decreased the production of mature neurons (BrdU+/NeuN+ cells) in the granule cell layer in adult female but not adult male rats, but (C) did not have any statistically significant effect on the production of young neurons (cells with processes containing DCX; $n = 3-4$ in each group), as indicated by the density of labelled cells per unit volume of the granule cell layer. Bars show group mean + standard error of the mean. * statistically significantly different ($p < 0.05$) from oil-injected controls.

Figure 5: Repeated estradiol administration decreased cell death in the granule cell layer of the dentate gyrus and subgranular zone in adult female but not adult male rats, as measured by cells expressing (A) a pyknotic morphology, (B) active caspase-3, or (C) degenerating neurons (cells immunoreactive with Fluoro-Jade B), as indicated by the density of cells per unit volume of the granule cell layer. Bars show group mean + standard error of the mean. * statistically significantly different ($p < 0.05$) from oil-injected controls.

Figure 6: Serum estradiol level in female ($\bullet$), but not male ($\diamond$), rats receiving oil or estradiol treatment varied with the density in the granule cell layer (per unit volume of the granule cell layer) of (A) cells labelled with BrdU, (B) the density of pyknotic cells in the granule cell layer of the dentate gyrus, and (C) the density of cells labelled with

Fluoro-Jade, but not with (D) the density of cells expressing Ki-67, (E) the density of cells expressing DCX, or (F) the density of cells expressing activated caspase-3.

GCL

SGZ

Hilus