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Research Article

Estradiol-Induced Enhancement in Cell Proliferation Is Mediated Through Estrogen Receptors in the Dentate Gyrus of Adult Female Rats

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ABSTRACT High-level estradiol enhances cell proliferation in the dentate gyrus of adult female rats within 4 h of administration and then suppresses cell proliferation within 48 h via an adrenal steroid-dependent mechanism (Ormerod et al., [2003b] J Neurobiol 55:247–260). Here, we investigate whether the estradiol-induced enhancement in progenitor cell proliferation is mediated through estrogen receptors (ERSs) using the selective ER antagonist ICI 182,780 (ICI). Ovariectomized Sprague-Dawley rats were given two subcutaneous injections of either vehicle + vehicle (VEH; 0.1 ml sesame oil); VEH+ICI (500 μg); estradiol benzoate (EB; 10 μg)+VEH; or EB +ICI. The cell synthesis marker, 5-bromo-2′-deoxyuridine (200 mg/kg) was administered 4 h later. Animals were perfused 24 h after BrdU injection and cell proliferation was assessed following immunohistochemical processing of the tissue. Relative to VEH, EB increased cell proliferation by approximately 50%. This EB-induced increase was partially blocked by ICI 182,780 treatment, and ICI 182,780 treatment alone enhanced cell proliferation. Our results demonstrate that estradiol enhances cell proliferation in the dentate gyrus of adult female rats by activating estrogen receptors. Drug Dev. Res. 66:142–149, 2006.

Key words: adult neurogenesis; estradiol; estrogen receptors; hippocampus; bromodeoxyuridine; cell proliferation

INTRODUCTION

After the original discovery by Altman in 1962, neurogenesis in the adult dentate gyrus has been observed across a wide variety of species [Cameron and McKay, 1999; Eriksson et al., 1998; Gould et al., 1998; Kornack and Rakic, 1999]. Precursor cells located in the subgranular zone divide, with many of the progeny becoming neurons that are incorporated into the granule cell layer of the dentate gyrus [Cameron et al., 1993]. Approximately 270,000 cells are produced every month, of which a substantive number become neurons [Cameron and McKay, 2001]. Although the behavioral function of adult hippocampal neurogenesis is not known, ablated hippocampal neurogenesis is associated with impaired learning and/or memory [Raber et al., 2004; Rola et al., 2004; Shors et al., 2001] and enhanced survival among young neurons promotes spatial memory [Ormerod et al., 2004]. Interestingly, senescent maze-impaired rats have fewer proliferating progenitors and surviving new neurons in their dentate gyri than controls [Drapeau et al., 2003]. Understanding the mechanisms that stimulate adult

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hippocampal neurogenesis could provide therapeutic avenues for treating age-related diseases that affect hippocampal function.

In rodents, estradiol influences neurogenesis in the dentate gyrus by affecting both the proliferation of progenitor cells and the survival of young neurons in rodents [Banasr et al., 2001; Ormerod et al., 2003a,b, 2004; Tanapat et al., 1999]. Progenitor cell proliferation is increased by a 4-h exposure to estradiol, but decreased by a 48-h exposure to estradiol in the dentate gyrus of the adult female rat [Ormerod et al., 2003b] and the meadow vole [Ormerod and Galea, 2001; Ormerod et al., 2003a]. Evidence suggests that the estradiol-induced suppression in cell proliferation occurs partially via an adrenal steroid-dependent mechanism [Ormerod et al., 2003b], and that the estradiol-induced stimulation of cell proliferation is mediated partially by serotonin [Banasr et al., 2001].

Current evidence suggests that estrogen exerts its effects through several different pathways: through non-genomic mechanisms, genomic mechanisms through the estrogen receptor (ER), and/or through an indirect molecular cascade of events that precipitates estradiol's effects on gene transcription. Nuclear and cytoplasmic ERs have been found in the hippocampus, including the subgranular zone where progenitor cells proliferate [Shughrue et al., 1997; Shughrue and Merchenthaler, 2000; Milner et al., 2001; Kalita et al., 2005]. There are at least two related proteins, estrogen receptor α (ERα) and estrogen receptor β (ERβ), along with its isoforms (ERβ1 and ERβ2) [Kuiper et al., 1996, 1997; Brannvall et al., 2002; Peterson et al., 1998; Maruyama et al., 1998], that mediate estrogen signalling in the brain. Ligand-bound estrogen receptors can alter gene expression by binding to classical estrogen response elements (EREs) or non-classical AP1 sites within a target gene [Webb et al., 1999]. Because direct genomic mechanisms, either via the ERE or AP-1 pathways, require in excess of 45 min, estradiol could increase progenitor cell proliferation within 4 h via these classic receptors. However, extranuclear ERs are also expressed in the hippocampus [Azcoitia et al., 1999; Kelly and Levin, 2001; Milner et al., 2001; Kalita et al., 2005] and they could influence progenitor cell proliferation through second messenger cascades or via translocation from the membrane to the nucleus. There are also several possible non-genomic mechanisms through which estradiol could act to alter cell proliferation. Such mechanisms include the phosphorylation of CREB [Levin, 1999; Kelly and Wagner, 1999; McEwen, et al., 2001], and the effects on cyclic AMP (cAMP) and mitogen-activated protein (MAP) kinase pathways that result from the actions of estrogens on neurotrophins such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 or (IGF-1) [Cardona-Gomez et al., 2000; Kaehlert et al., 2000; Kelly and Levin, 2001; Perez-Martin et al., 2003]. Intriguingly, progenitor cells harvested from the fetal and adult rodent subventricular zone (SVZ) express both estrogen receptor subtypes, ERα and ERβ, and estradiol stimulates the proliferation of SVZ-derived progenitors in vitro [Brannvall et al., 2002]. This result indicates that either subtype of ER could directly influence progenitor cells to initiate division, although a recent report by Tanapat et al., [2005] suggests that ERs are not located on progenitor cells located in the subgranule zone. However, those authors failed to find ERβ in the dentate gyrus contrary to previous literature [Kalita et al., 2005; Shughrue and Merchenthaler, 2000].

As discussed above, estradiol may mediate its effects directly by stimulating ERs on hippocampal progenitor cells, or indirectly, by stimulating ERs on cells located within the neurogenic niche. The present study investigated whether estradiol stimulates hippocampal progenitor cell proliferation by acting through ERs. Adult female rats were exposed to estradiol alone, the estrogen receptor antagonist ICI 182,780 alone, or the combination of estradiol and ICI 182,780 prior to assays of cell proliferation using the cell synthesis substrate bromodeoxyuridine. ICI 182,780 is a pure ERα antagonist and a partial ERβ antagonist [Webb et al., 1999, 2003], and it is considered to be a pure estrogen receptor antagonist at EREs sites for both ERα and ERβ, but only at AP-1 sites where ERα mediates gene transcription. ICI 182, 780 appears to be an *agonist* of ERβ-mediated gene transcription at AP-1 sites because ERβ enhances AP-1 activity in the presence of ICI compounds [Paech et al., 1999; Webb et al., 1999, 2003]. Therefore, we hypothesized that if the estradiol-induced cell proliferation was eliminated by ICI 182,780, then this process could be mediated by estradiol working through ERα and ERβ to enhance gene activity at EREs or through ERα to enhance AP-1 activity. Alternatively, an enhancement in cell proliferation induced by ICI 182,780 treatment alone could suggest that this process is mediated though ERβ to enhance AP-1 activity.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley (290–350 g) rats were obtained from Charles River Laboratories (Canada; n = 4–5 per group). Animals were housed singly in polyurethane cages in a colony room with a 12:12 h light dark cycle (lights on at 0800 h) and

were given access to food (Purina Lab Diet 5012) and water ad libitum. All research was conducted in accordance with the guidelines of the Canadian Council on Animal Care and the policies of the University of Toronto. Every effort was made to minimize the suffering of animals, and to minimize the number of animals used.

Approximately 1–2 weeks after arrival, all animals were bilaterally ovariectomized using Isofluorane to induce anaesthesia (approximately 3% flow). Isofluorane was reduced on an individual basis (1.5–2.5% flow rate) throughout the surgery, and respiration was continually monitored. Animals were allowed to recover for one week prior to the commencement of testing.

Ovariectomized (OVX) adult female Sprague-Dawley rats were each given two separate (one immediately following the other) 0.1-ml subcutaneous injections composed of either vehicle + vehicle (sesame oil; VEH+VEH); vehicle + ICI (500 μg per rat; VEH+ICI); 17β-estradiol benzoate (EB; 10 μg per animal) + VEH; or EB + ICI. The cell synthesis marker BrdU (200 mg/kg) was administered intraperitoneally 4 h after the two subcutaneous injections. Rats were perfused 24 h after administration of BrdU.

Compounds

BrdU (Sigma Aldrich Chemicals) was prepared to a concentration of 20 mg/ml in warm fresh saline (9%) buffered with 7 μl of 2N NaOH per ml of saline. 17β-estradiol benzoate (Sigma Aldrich Chemicals) was dissolved in sesame oil (Sigma Aldrich Chemicals) over low heat to obtain a concentration of 10 μg EB per 0.1 ml sesame oil [Ormerod et al., 2003b]. The solution was mixed and stored in light-insensitive containers. ICI 182, 780 (Tocris Chemicals) was also dissolved in sesame oil over low heat. A dose of 500 μg per 0.1 ml sesame oil was prepared.

Histology

Rats were perfused with 4% paraformaldehyde and the brains stored for 48 h in perfusate at 4°C. Then, the brains were sliced through the extent of the entire dentate gyrus in a bath of tris-buffered saline (TBS; PH 7.5) using a Leica VT1000S oscillating tissue slicer. The 40-μm-thick sections were stored in TBS at 4°C until immunohistochemistry was performed.

BrdU Immunohistochemistry

All tissue was rinsed repeatedly using TBS (pH 7.5) between each step unless otherwise specified. Sections were incubated in 0.3% hydrogen peroxide (H_2O_2) for 10 min to quench endogenous peroxidase, then incubated in formamide solution for 2 h at 65°C, rinsed in SSC, and incubated in 2M HCl for 30 min at 37°C to denature DNA. The sections were then neutralized in 0.1 M borate buffer (pH 8.5), blocked in TBS^{++} (TBS + 0.3% Triton-X [Sigma Chemicals], 5% normal horse serum [Vector S-2000]) and incubated for 72 h in mouse monoclonal anti-BrdU (Boehringer Mannheim; 1:500 in TBS^{++}) at 4°C. The sections were rinsed repeatedly and then incubated for 4 h in anti-Mouse IgG (Vector Elite ABC Kit PK6102), and then for 2 h in AB Reagent (avidin-biotin horseradish peroxidase complex (Vector Elite ABC Kit PK6102). They were then reacted in 3,3′-diaminobenzidene and mounted on 3-aminopropyltrieth-oxysilane- (Sigma Chemicals) treated slides. The slide-mounted sections were dried overnight before counterstaining with cresyl violet (Acros Organic-Fisher Scientific) and attaching cover-slips with permount.

Stereological Measurements

The slides were coded prior to counting to keep the investigator blinded to the experimental conditions. BrdU-labelled cells were counted using the 100 × objective on a Nikon Eclipse (E600) Microscope. Five sections per brain, taken every 20th section throughout the dentate gyrus, were analyzed. Intensely labelled BrdU-positive cells in the granule cell layer and subgranular zone (area of approximately 50 μm between hilus and granule cell layer [Palmer et al., 2000]) were counted if they exhibited oval or medium round cell bodies [Cameron et al., 1993] (see Fig. 1). An estimate of the total cell count was calculated by multiplying the number of BrdU-labelled cells × 20.

The area of the dentate gyrus was measured using Simple PCI (Nikon Canada) and the volume of the dentate gyrus (granule cell layer and subgranular zone) was estimated using Cavalieri's principle [Gunderson et al., 1988]. Density measurements were created by dividing the number of BrdU-labelled cells by the combined area of the dentate gyrus and subgranular zone.

Confocal laser microscopy to confirm the phenotype of BrdU-ir cells was not utilized in the present study because animals were perfused 24 h after BrdU injection. This amount of time is sufficient to observe one mitotic division among progenitor cells [Cameron and McKay, 2001], but it is too short to allow for the detection of immature neuronal proteins using antibodies like anti-doublecortin or anti-TUC-4 among most daughter cells. Previous work, utilizing longer time lapses (at least 48–96 h) before perfusion, found that the majority of new cells labelled with BrdU in the dentate gyrus of adult female rats following EB treatment expressed markers for immature and mature neurons [Ormerod et al., 2003b; Tanapat et al., 1999].

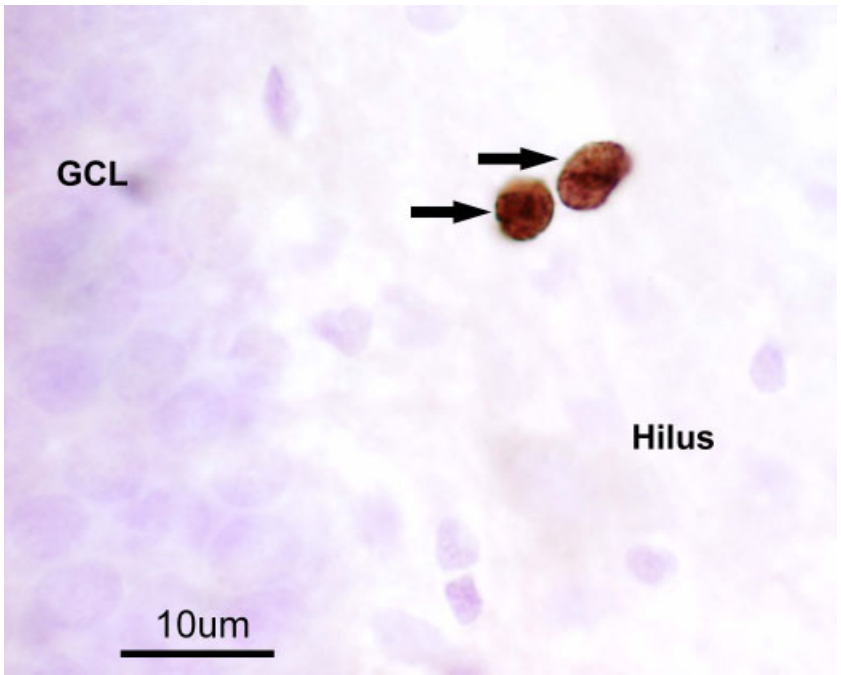

Fig. 1. Photomicrograph of BrdU-labelled cells (indicated by the arrows) in the subgranular zone of the dentate gyrus. Scale bar = 10 μm. GCL, granule cell layer.

Statistical Analyses

The density of BrdU-immunoreactive (ir) cells and the volume of the dentate gyrus (dependent variables) were each analyzed using an analysis of variance (ANOVA) with the hormone group (VEH or EB) and the ICI group (VEH or ICI 182, 780) as between-subjects factors. All statistical procedures were set at $\alpha = 0.05$. For post-hoc testing, Fisher's LSD (least significant difference) was utilized unless otherwise specified.

RESULTS

We used a density measure for BrdU-labelled cells to avoid potential differences in volumes counted accounting for group differences. With this approach, we found that a 4-h exposure to high-level estradiol significantly increased the density of BrdU-labelled cells in the dentate gyri of adult female rats, relative to vehicle ($P \leq 0.01$). There was a significant interaction effect [$F(1,14) = 10.36$, $P \leq 0.006$] (see Fig. 2), whereas the main effects were not significant (hormone [$P \leq 0.34$] or ICI [$P \leq 0.84$]). The increase in density with estradiol was partially blocked by ICI 182,780 treatment, as there was no significant difference in number of BrdU-labelled cells observed between the VEH+VEH and EB+ICI groups ($P < 0.39$), and the EB+VEH group tended to have more BrdU-labelled

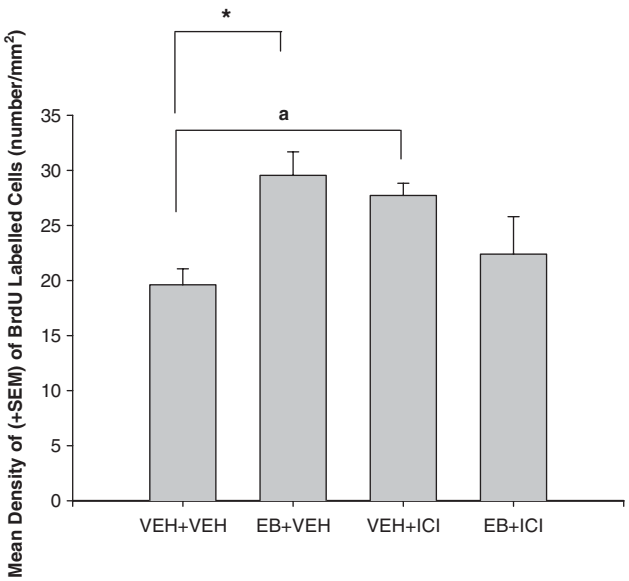

Fig. 2. Mean (±SEM) number of new cells in the dentate gyrus of adult female rats given BrdU 4 h after two separate injections of either vehicle + vehicle (VEH + VEH); estradiol + vehicle (EB + VEH); estradiol + ICI 182, 780 (EB + ICI); or vehicle + ICI 182, 780 (VEH + ICI). Estradiol significantly increased the mean density of new (BrdU-ir) cells observed in the dentate gyrus of adult female rats within 4 h of injection when compared to the vehicle group. ICI 182,780 alone significantly elevated BrdU-ir cells and ICI 182,780 combined with EB partially blocked the estradiol-induced enhancement of BrdU-ir cells. n = 4, 5 per group. Asterisk (*) indicates statistical significance at $P \leq 0.05$; a indicates $P \leq 0.052$.

cells when compared with the EB+ICI group ($P \leq 0.052$). Interestingly, the analysis also revealed an increase in the density of BrdU labelled cells in the VEH+ICI group compared with the VEH+VEH group ($P \leq 0.03$), suggesting an effect of ICI 182,780 alone to enhance cell proliferation.

While there was a slight trend for a significant main effect of estradiol ($F(1,14) = 3.7$; $P \leq 0.08$; see Table 1) on dentate gyrus volume, there was no significant main effect of the ICI compound ($P \leq 0.18$), or an interaction effect ($P \leq 0.47$), on dentate gyrus volume. Thus, volume is not as sensitive as test parameter as density (number of BrdU-labelled cells/area).

DISCUSSION

In the present study, BrdU-labelled cell density, our measure of cell number, was significantly increased in the dentate gyri of estradiol-treated rats relative to vehicle-treated rats, consistent with previous studies [Ormerod and Galea, 2001; Ormerod et al., 2003a,b; Tanapat et al., 1999, 2005]. ICI 182,780, an ER antagonist, partially blocked the estradiol-induced enhancement of cell proliferation and it also increased the BrdU-labelled cell number when used alone.

Estradiol Acts Via Estrogen Receptors to Increase Cell Proliferation

The findings from the present study suggest that estradiol enhances progenitor cell proliferation in the dentate gyri of adult female rats, at least in part, through ER-mediated pathways, because ICI 182,780 partially reversed the estradiol-induced enhancement. Because ICI 182,780 is a full antagonist of ERα and a partial antagonist of ERβ, estradiol could mediate its effects through either receptor. However, the observation that ICI 182,780 alone increased cell proliferation suggests that ERβ plays a role in increasing progenitor cell proliferation.

There are several possible non-receptor-mediated mechanisms of estradiol that could explain the partial, rather than complete, reversal by ICI 182, 780 of the estradiol-induced enhancement of cell proliferation. The mechanisms include the following:

1. CREB Phosphorylation [Levin, 1999; Kelly and Wagner, 1999];
2. Effects on cyclic AMP and mitogen-activated protein kinase (MAP kinase) pathways that result from the actions of estradiol on neurotrophins such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) and their receptors (tyrosine-related kinase receptors (trk) [Cardona-Gomez, 2000; Kahlert et al., 2000; Kelly and Levin, 2001];
3. Effects on calcium currents and gonadotropin-releasing hormone (GnRH) release resulting from effects on G protein–coupled receptors [Kelly and Levin, 2001; Razandi et al., 2003];
4. Neuroprotection from free radicals and excitotoxins [Garcia-Segura et al., 2001].

Support for a non-genomic mechanism of estradiol comes from the work of Singh et al. [1999] who found that EB treatment rapidly activates MAP kinases via a mechanism that is not blocked by ICI 182,780. In addition, Perez-Martin et al. [2003] found that ICI 182,780 blocks the IGF-1 induced enhancement in neurogenesis in the dentate gryus, suggesting that IGF-1 can work through the estrogen receptors to mediate its effects on neurogenesis. Thus, the current evidence suggests that estradiol may exert its effects on cell proliferation in the dentate gyrus by several different pathways: (1) through ER-mediated genomic and non-genomic pathways; and/or (2) through an indirect molecular cascade of events that involves estradiol's effects on gene transcription. These indirect estrogenic actions are likely to include effects on neurotrophins [Cardona-Gomez, 2000; Kahlert et al., 2000; Kelly and Levin, 2001; Perez-Martin et al., 2003], such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1), and on their receptors, including tyrosine-related kinase receptors.

Both ERα and ERβ Are Involved in the Upregulation of Cell Proliferation

In the current study, ICI 182, 780 upregulated cell proliferation alone suggesting that the ERβ may be involved in upregulating hippocampal cell proliferation. Integral to this interpretation is the idea that transactivational functions and coregulators play a central role in estrogen receptor action at AP-1 sites that is strikingly different from what occurs at the ERE [Webb et al., 1999, 2003]. The expression of genes that contain AP-1 sites (cognate binding site for Jun/Fos complex), or other binding sites for transcription factors (such as related cyclic AMP response elements), is enhanced through interactions with estrogen receptors [Paech et al., 1997; Webb et al., 1995, 1999]. Indeed, several studies have demonstrated opposing effects of ERα and ERβ in response to estradiol at AP-1 sites: ERα enhances transcriptional activity, whereas ERβ inhibits it [Liu et al., 2002; Paech et al., 1999; Webb et al., 1995, 1999]. Taken together with evidence suggesting that ICI 182, 780 acts as an *agonist* at AP-1 sites regulated by ERβ [Paech et al., 1997; Webb et al.,

1999], the results of the current study are consistent with the idea that the suppression in estradiol-induced increase in cell proliferation by ICI 182, 780 may have been mediated through the ERα at an ERE or AP-1 site (and possibly ERβ at an ERE), while the enhancement of cell proliferation by ICI 182, 780 alone is suggestive of ERβ involvement at an AP-1 site. Further evidence that ERα and ERβ are involved in the upregulation of cell proliferation comes from recent evidence from our laboratory showing that selective ERα and ERβ agonists both enhanced cell proliferation in the dentate gryus (Mazzucco et al., unpublished observations). Curiously, progenitors cells do not appear to express ERα or β in the dentate gyrus, suggesting a non-direct stimulation of cell proliferation [Tanapat et al., 2005]. However, Tanapat et al. [2005] did not find evidence of ERβ in the dentate gyrus contrary to previous reports [Kalita et al., 2005].

Alternative Mechanisms for Estradiol-Induced Enhancement in Cell Proliferation That May Be Working in Conjunction With ERs

Alternative explanations for the estradiol-induced enhancement in cell proliferation observed can be found in several recent in vitro studies that provide evidence for the localization of known estrogen receptor activity in the plasma membrane of cells in the central nervous system (CNS), including the hippocampus [Azcoitia, 2003; Kelly and Levin, 2001; Milner et al., 2001; Razandi et al., 1999, 2003]. Evidence to suggest that membrane-associated forms of ERα and ERβ can act through second messenger signalling and G-protein coupling is intriguing [Kelly and Levin, 2001; Razandi et al., 1999, 2003], as it is well known that growth factors stimulate cell proliferation through the activation and translocation of the mitogen-activated protein kinase, extracellular regulated kinase (ERK) [Razandi et al., 1999; Wade and Dorsa, 2003]. MAPK activation by membrane ERs was also observed in a study by Wade and colleagues [2001] who found that in rat-2 fibroblasts transfected with either human ERα or rat ERβ clones, ICI 182,780 was able to block MAPK activation by ERα, but not ERβ. In addition, support for the existence of ERα in the nuclear membrane of both pyramidal cells and granule neuron perikarya in the hippocampus was put forth by Milner et al. [2001], suggesting that ERs may play both a genomic and non-genomic role in the hippocampus, possibly by stimulating signal transduction through G protein–coupled ERs.

Another plausible hypothesis for the observed estradiol-induced enhancement of cell proliferation in the dentate gyrus is through increased gene expression of astroglia [Azcoitia, 2003]. These astrocytes could, in turn, enhance neurogenesis via their synthesis and release of growth factors [Song et al., 2002]. This idea is compelling when one contemplates the evidence suggesting that astrocytes can substantially enhance progenitor cell proliferation [Song et al., 2002], in conjunction with evidence suggesting that there is an increase in astrocyte surface density in the dentate gyrus during proestrus [Luquin et al., 1993], the time frame coincident with the estradiol-induced increase in cell proliferation [Tanapat et al., 1999]. Intriguingly, recent findings indicate that both ERα [Milner et al., 2001; but see Azcoitia et al., 2003] and ERβ immunoreactive cells [Azcoitia et al., 2003] have been detected in astrocytes within the hippocampus, possibly indicating a mechanism by which estradiol exerts its influence on cell proliferation. Furthermore, brain endothelial cells express ERα [Stirone et al., 2003], and because progenitor cells proliferate in tight association with vasculature [Palmer et al., 1999], estradiol might act in association with endothelial cells to influence cell proliferation in the dentate gyrus.

Conclusions

Overall, this study provides insight into the mechanisms by which hippocampal neurogenesis is dynamically affected by estradiol. The present work supports previous research demonstrating that a 4-h exposure to high-level estradiol enhances progenitor cell proliferation [Ormerod and Galea, 2001; Ormerod et al., 2003b; Tanapat et al., 1999], and, importantly, it provides evidence that this increase is mediated, at least in part, by estrogen receptors. Indeed, the results suggest that both ER subtypes α and β are likely to be involved in this complex process. Future studies are needed to specifically determine the location and type(s) of estrogen receptor being utilized, and to understand the possible synergistic effects of estradiol, neurotrophins, and second messenger systems in the adult female rat hippocampus. Of note, this work has also raised questions about the specific effects of

TABLE 1. Mean (±SEM) Volume of Dentate Gyrus of Ovariectomized Adult Female Rats Given BrdU 4 h After Injection of Vehicle+Vehicle, Estradiol+Vehicle, Estradiol+ICI 182, 780, or Vehicle+ICI 182, 780[a]

Groups	Dentate gyrus volume (mm^2)
VEH+VEH	0.53±0.02
VEH+ICI	0.49±0.02
EB+VEH	0.56±0.02
EB+ICI	0.55±0.01

[a]Mean (±SEM) dentate gyrus volume of adult female rats given BrdU 4 h after two separate injections of either vehicle+vehicle (VEH+VEH); estradiol+vehicle (EB+VEH); estradiol+ICI 182, 780 (EB+ICI); or vehicle+ICI (VEH+ICI). There was no significant difference between groups on dentate gyrus volume.

the estrogen receptor antagonist ICI 182,780 in the rodent brain. Together, these results bring us one step closer to understanding the multifaceted effects of estradiol and estrogen receptors in neurogenesis. It is hoped that such insight will inspire therapeutic innovation in devastating degenerative illnesses such as Alzheimer's Disease.

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