Estrogens dynamically regulate neurogenesis in the dentate gyrus of adult female rats

Shunya Yagi[a], Yanhua Wen[b] and Liisa AM Galea[a,b,c]

[a] Graduate Program in Neuroscience, University of British Columbia, Vancouver, (BC) Canada
[b] Department of Psychology, University of British Columbia, Vancouver, (BC) Canada
[c] Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver, (BC) Canada

Short Title: **Estrogens regulate adult hippocampal neurogenesis**

Corresponding Author:

Liisa Galea, PhD
Djavad Mowafaghian Centre for Brain Health
University of British Columbia
2215 Wesbrook Mall
Vancouver, British Columbia, V6T 1Z3, Canada
Tel: +1 (604) 822 3941
Fax: +1 (604) 822 6923
Email: liisa.galea@ubc.ca

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Abstract

Estrone and estradiol differentially modulate neuroplasticity and cognition but how they influence maturation pathways of new neurons is not known. The present study assessed the effects of estrone and estradiol on various aspects of neurogenesis in the dentate gyrus (DG) of ovariectomized young adult Sprague-Dawley rats using daily subcutaneous injections of 17β-estradiol or estrone. Rats were injected with a DNA synthesis marker, 5-bromo-2-deoxyuridine (BrdU), and were perfused one, two, or three weeks after BrdU injection and treatment. Immunofluorescent labelling for Sox2 and Ki67 were used to examine the density of neural stem cells and proliferating cells, respectively. Double-immunofluorescent labelling of BrdU with doublecortin (DCX) or NeuN was used to examine the attrition and maturation of adult-born neurons over time. Estradiol reduced the density of neural stem cells in the dorsal DG, whereas estrone reduced the density of neural stem cells in the ventral DG. Furthermore, estradiol enhanced, whereas estrone reduced, cell proliferation after one week but not after longer exposure to hormones. Both estrogens increased the density of BrdU/DCX-ir cells after one week of exposure but showed greater attrition of new neurons between one and two weeks after exposure. Lastly, estradiol decreased the percentage of BrdU/NeuN-ir cells in the dorsal DG after three weeks of treatment. These results demonstrate that estrogens have differential effects to modulate several aspects of adult hippocampal neurogenesis in the short term, but fewer effects after long-term exposure and that estradiol and estrone modulate neurogenesis via different pathways.

1. Introduction

Hippocampal integrity is compromised in diseases such as Alzheimer's disease and depression [1,2]. A unique characteristic of the hippocampus is its ability to generate new neurons in adulthood. Adult neurogenesis in the hippocampus plays important roles for pattern separation during memory encoding and in stress resilience [3–5]. New neurons in the adult hippocampus are produced from neural stem cells in the subgranular zone of dentate gyrus (DG). Developing new neurons express stage-specific endogenous markers such as Sox2 in neural stem cells, Ki67 in proliferating cells, doublecortin (DCX) in immature neurons, and neuronal nuclei (NeuN) in mature neurons [6]. Although there are no sex differences in the number of new three-week old neurons in rats, there are sex differences in the maturation pathways for adult neurogenesis [7]. Male rats have a greater density of neural stem cells, greater cell proliferation, and faster maturation of new neurons than female rats [7]. However, male rats also have greater attrition of immature neurons between one and two weeks after production compared to female rats [7]. These findings suggest it may be fruitful to determine whether ovarian hormones, such as estrogens, regulate the maturation of neurogenesis in female rodents.

There are four types of estrogens, estrone, estradiol, estriol and estetrol. Estrone and estradiol are the two most abundant of the estrogens. Estradiol binds with greater affinity to estrogen receptors (ERs) and is present at higher levels than estrone before menopause whereas estrone is present at higher levels than estradiol after menopause in human females [8]. Previous studies demonstrate that estrogens modulate cell proliferation in the dentate gyrus which depends on both the type of estrogens and duration of exposure [9–13]. A single dose of 17β- estradiol or estradiol benzoate (EB) rapidly increases cell proliferation but this effect depends on the duration of exposure and formulation of estradiol [9–11,13]. However, repeated administration of

estradiol or EB for three weeks had no significant effect on cell proliferation [12,14,15]. Collectively these studies suggest that the duration of exposure to estradiol can dramatically influence the effects on cell proliferation in the dentate gyrus.

Estrone treatment has different effects on neurogenesis in the hippocampus and contextual fear conditioning than estradiol in female rats [14,16]. Although both estrogens increase cell proliferation [9], estradiol enhances, whereas estrone decreases, survival of three-week old new neurons in rats that also underwent cognitive training [14]. In addition, acute exposure to estradiol enhances, whereas estrone impairs, contextual fear conditioning in adult female rats [16]. These studies suggest that these different estrogens have differential effects on different aspects of neurogenesis and hippocampus-dependent function.

Estrogens also influence cognition and hippocampal volume in humans depending on the type, timing and duration of hormone therapy (reviewed in [17,18]). For instance, short duration of hormone therapy increases hippocampal volume [19,20], whereas longer than ten years of hormone therapy decreases hippocampal volume in postmenopausal females [19]. Furthermore, estradiol-based hormone therapy improves verbal memory in post-menopausal females, whereas conjugated estrone-based hormone therapy has detrimental (or no significant) effects on verbal memory [21–26]. The timing of initiation of hormone therapy relative to menopause also plays important roles for the effects of hormone therapy as early initiation after menopause enhances cognitive performance, whereas late initiation after menopause leads to poorer performance [27], an effect mirrored in animal models (reviewed in [28]).

To date, duration-dependent changes in the effects of estrogens have not been investigated on the characteristics of neurogenesis in the DG. Thus, we aimed to elucidate effects of estrone and estradiol on neural stem cells, maturation rate of new neurons, and the trajectory

(attrition) of new neurons. We hypothesized that estradiol and estrone would differentially modulate the trajectory and maturation rate of new neurons based on the duration of exposure to estrogens.

2. Materials and Methods

2.1. Subjects

Thirty-six female Sprague-Dawley rats (four females each treatment and each maturation time courses) obtained from our breeding colony at University of British Columbia (Vancouver, BC, Canada) were used in this study. Rats were weaned at postnatal day 21 and housed with same-sex siblings until puberty. Rats were then pair-housed until the end of the study in opaque polysulfone bins (432 mm × 264 mm × 324 mm) with paper towels, a single polycarbonate hut, virgin hardwood chip bedding, and free access to food and water. The colony room was maintained under a 12:12-h light/dark cycle (lights on at 07:00 h). All experiments were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and their suffering during all procedures.

2.2. Experimental timeline

Rats were handled for 2 minutes every day beginning at the age of ten weeks for two weeks. Rats received ovariectomy bilaterally at the age of twelve weeks and rats were randomly assigned into three treatment groups. Following one week of recovery period, rats started to receive 5 μg Estrone in 0.1 ml sesame oil, 5 μg of 17β in 0.1 ml sesame oil or 0.1 ml sesame oil (vehicle) via subcutaneous injection. Daily subcutaneous injections of 5 μg of 17β results in serum concentrations of estradiol equivalent to the levels of estradiol during proestrous phase [29]. Each group received the same treatment every day (approximately 9-11 am) until the end of

experiment. On the next day, all rats received one injection of bromodeoxyuridine (BrdU; 200 mg/kg i.p.) one hour after hormone or vehicle treatment. Rats were perfused one, two or three weeks after BrdU injection (Figure 1A). Serum estradiol levels were 1.59x times higher in the estradiol group versus the estrone group which were 50x and 20x higher than the oil injected groups (verified via a multiplex electrochemiluminescence immunoassay kit (Custom Steroid Hormone Panel, Human/Mouse/Rat) from Meso Scale Discovery (Rockville, MD, USA)).

2.3. Perfusion and tissue processing

Rats were administered an overdose of sodium pentobarbitol (500 mg/kg, i.p.) and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% formaldehyde (Sigma-Aldrich). Brains were extracted and post-fixed in 4% formaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution for cryoprotection and remained in the solution until sectioning. Brains were sliced into 30 μm coronal sections using a Leica SM2000R microtome (Richmond Hill, Ontario, Canada). Sections were collected in series of ten throughout the entire rostral-caudal extent of the hippocampus and stored in anti-freeze solution consisting of ethylene glycol, glycerol and 0.1M PBS at -20°C.

2.4. Immunohistochemistry

Brain sections were stained for Sox2 (Figure 1B) and Ki67 (Figure 1E) to examine the density of neural stem cells or the density of proliferating cells in the DG, respectively. Furthermore, brain sections were double-stained for BrdU/DCX (Figure 2A-C) and BrdU/NeuN (Figure 2D-F) to examine the maturation time course of new cells. Furthermore,

2.4.1. BrdU/NeuN or BrdU/DCX double-labelling

Brain sections were prewashed three times with 0.1 M PBS and left overnight at 4 °C. The tissue was incubated in a primary antibody solution containing 1:250 mouse anti-NeuN

(Milli- pore; MA, USA) or 1:200 goat anti-DCX (Santa Cruz Biotechnology, CA, USA), 0.3% Triton-X, and 3% normal donkey serum (NDS; Vector Laboratories) in 0.1 M PBS for 24 hours at 4 °C. Following three rinses in 0.1 M PBS, sections were incubated in a secondary antibody solution containing 1:200 donkey anti-mouse Alexa Fluor 488 (Invitrogen, Burlington, ON, Canada) or 1:200 donkey anti-goat Alexa Fluor 488 (Invitrogen, Burlington, ON, Canada) in 0.1 M PBS, for 18 hours at 4 °C. After rinsing three times with PBS, the sections were washed with 4% formaldehyde, and rinsed twice in 0.9% NaCl, followed by incubation in 2N HCl for 30 minutes at 37 °C. Following three rinses in 0.1 M PBS, the sections were then incubated in a BrdU primary antibody solution consisting of 1:1000 rat anti-BrdU (AbD Serotec; Raleigh, NC, USA), 3% NDS, and 0.3% Triton-X in 0.1 M PBS for 24 hours at 4 °C. Sections were then incubated in a secondary antibody solution containing 1:500 donkey anti-rat Cy3 (Jackson ImmunoResearch; PA, USA) in 0.1 M PBS for 24 hours at 4 °C. Following three rinsed with PBS, the sections were mounted onto microscope slides and cover-slipped with PVA DABCO.

2.4.2. Sox2

Brain sections were prewashed with 0.1 m PBS and left to sit overnight at 4°C. The next day, sections were washed in 0.1M PBS for 10 min each and blocked with 3% NDS and 0.3% Triton X-100 in 0.1 M PBS, followed by incubation in primary antibody solution made with 1:1000 mouse anti-Sox2 (Santa Cruz Biotechnology), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS for 24 h at 4°C. Then the sections were incubated in secondary antibody solution, consisting of 1:500 donkey anti-mouse Alexa Fluor 594 (Invitrogen), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS, for 18 h at 4°C. After three rinses with PBS, the sections were incubated in 1:5000 DAPI in PBS for 3 min. Followed by three rinses, tissues were mounted onto slides and cover-slipped with PVA DABCO.

2.4.3. Ki-67

Brain sections were prewashed with 0.1 M PBS and left to sit overnight at 4°C. The next day, sections were incubated in 10 mM sodium citrate buffer for 30 min at 90°C to retrieve antigens of Ki67 and blocked with 3% NDS and 0.3% Triton X-100 in 0.1 M PBS. Tissue was then incubated in primary antibody solution made with 1:250 mouse anti-Ki67 (Leica Biosystems), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS for 24 h at 4°C. Following three washes in 0.1 M PBS, brain sections were incubated in secondary antibody solution, consisting of 1:500 donkey anti-mouse Alexa Fluor 488 (Invitrogen), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS, for 18 h at 4°C. After three rinses with PBS, sections were incubated in 1:5000 DAPI in PBS for 3 min. Followed by three rinses, tissue was mounted onto slides and cover-slipped with PVA DABCO.

2.5. Cell counting

All counting was conducted by an experimenter blind to the group assignment of each animal using an Olympus FV1000 confocal microscope and/or Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy, Thornwood, NY, USA). Density of immunoreactive (ir) cells was calculated by dividing the total number of ir cells by volume (mm^3) of the corresponding region. Volume estimates were calculated by multiplying the summed areas by thickness of sections (0.03 mm, using Cavalieri's principle; [30]). Area measurements for the region of interest were obtained using digitized images on Zen 3.0 software (blue edition; Carl Zeiss Microscopy, Thornwood, NY, USA). Cells were categorized as to whether they were in the dorsal or ventral DG using the criterion defined by Banasr and others (2006), with sections 6.20-3.70 mm from the interaural line defined as dorsal and sections 3.70-2.28 mm from the interaural line as ventral. Cells were counted separately in each region because the dorsal hippocampus is associated with spatial

learning and memory, whereas the ventral hippocampus is associated more with stress and anxiety [31,32].

BrdU-ir cells were counted under a 60x oil immersion objective lens using an Olympus epifluorescent microscope and the percentages of BrdU/NeuN-ir cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that double-labelled-ir with NeuN under 40x objective lens using an Olympus FV1000 confocal microscope (Olympus, Richmond Hill, ON, Canada). The percentages of BrdU/DCX-ir cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that double-labelled-ir with DCX on digitized images acquired under 40x objective lens using Axio Scan.Z1 slidescanner with Zen 3.0 software (blue edition; Carl Zeiss Microscopy, Thornwood, NY, USA). Ki67-ir cells were counted on digitized images every twentieth section. Photomicrographs for Ki67-ir cells were taken with a 40x objective lens on a Axio Scan.Z1 slidescanner with Zen 3.0 software (Carl Zeiss Microscopy, Thornwood, NY, USA). Photomicrographs for Sox2-ir cells were taken from four dorsal and three ventral hippocampi using a 40x objective lens on Axio Scan.Z1 slidescanner, and optical density of Sox2-ir cells were measured on digitized images using ImageJ (NIH, Bethesda, MD, USA).

2.6. Statistical analyses

All analyses were conducted using STATISTICA (Statsoft Tulsa, OK). Repeated-measures ANOVAs were used to each analyze the density of Ki67-ir and Sox2-ir cells with exposure time (1w, 2w, 3w) and hormone (estrone, estradiol, vehicle) as between subject factor and with hippocampal region (dorsal, ventral) as the within-subject factor. The density of BrdU-ir cells or the percentage of BrdU/DCX-ir cells or BrdU/NeuN-ir cells were each analyzed using repeated-measures analysis of variance (ANOVA), with week (1w, 2w, 3w) and hormone

(estrone, estradiol, vehicle) as between-subject variables and with hippocampal region (dorsal, ventral) as the within-subject variable. Post-hoc tests utilized the Neuman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections. Significance was set to $\alpha=0.05$ and effect sizes are given with Cohen's d or partial η^2.

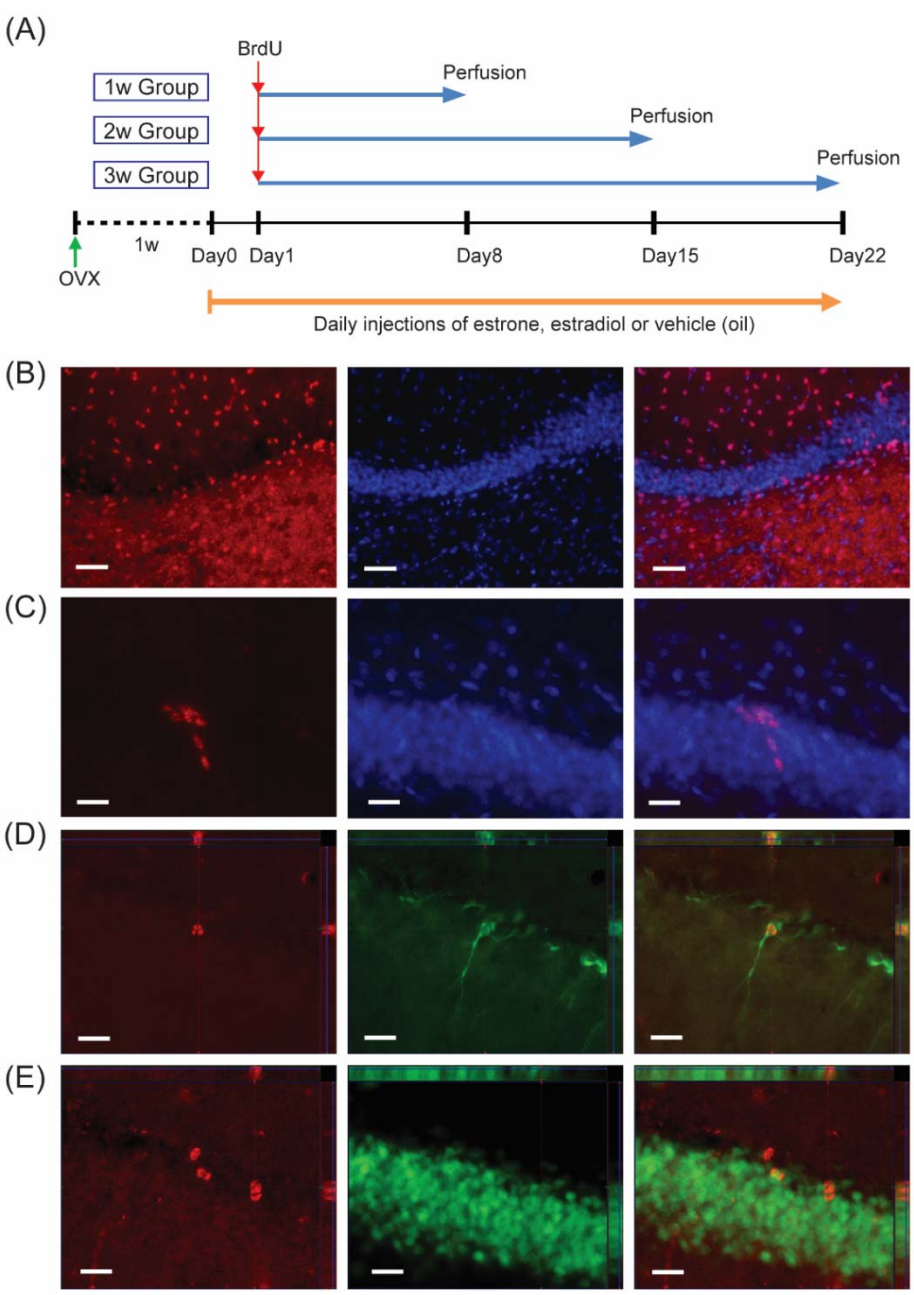

Fig. 1. A: Schematic illustration for the experimental timeline. **B-E:** Photomicrographs for (B) Sox2-ir cells (red) with DAPI (blue), (C) Ki67-ir cells (red) with DAPI (blue), (D) BrdU-ir cells (red) with DCX-ir cells (green) and (E) BrdU-ir cells (red) with NeuN-ir cells (green). Scale bars in (B) indicate 50 µm and scale bars in (C)-(E) indicate 20 µm. All photomicrographs were taken by Zeiss Axio Scan.Z1 with 20x (B) or 40x (C-E) objectives.

3. Results

3.1. Estradiol reduced the density of Sox2-ir cells in the dorsal DG, whereas estrone reduced the density of Sox2-ir cells in the ventral DG compared to vehicle-treated females.

Both estrogens reduced the density of Sox2-ir cells dependent on region but not on exposure time. Estradiol-treated females had a lower density of Sox2-ir cells in the dorsal DG compared to vehicle-treated females ($p = 0.021$, Cohen's $d = 1.222$), whereas estrone-treated females had a lower density of Sox2-ir cells in the ventral DG compared to both groups (vehicle-treated: $p = 0.006$, Cohen's $d = 1.410$; estradiol-treated: $p = 0.002$, Cohen's $d = 1.000$) [interaction effect of region by hormone: $F(2, 25) = 5.60$, $p < 0.01$, partial $\eta^2 = 0.309$: Figure 2A and 2B]. Furthermore, there was a greater density of Sox2-ir cells in the ventral DG compared to the dorsal DG in estradiol-treated ($p < 0.001$, Cohen's $d = 1.909$) and vehicle-treated females ($p = 0.01$, Cohen's $d = 0.959$) but not in estrone-treated females ($p=0.53$). There was also a main effect of hormone [$F(1, 25) = 4.19$, $p = 0.027$, partial $\eta^2 = 0.251$] and region as expected [$F(1, 25) = 29.36$, $p < 0.001$, partial $\eta^2 = 0.540$]. There were no other significant main or interaction effects on the density of Sox2-ir cells ($p > 0.180$).

3.2. Estradiol increased, whereas estrone reduced, the density of Ki67-ir cells in the DG compared to vehicle-treated females after one week of hormone exposure.

After one week of hormone treatment, estradiol-treated females had a greater density of Ki67-ir cells compared to both groups (vehicle-treated: $p < 0.001$, Cohen's $d = 2.864$; estrone-treated: $p < 0.001$, Cohen's $d = 5.239$), whereas estrone-treated females had a lower density of Ki67-ir cells compared to both groups (vehicle-treated: $p = 0.026$, Cohen's $d = 1.883$) [interaction effect of exposure time by treatment: $F(4, 26) = 10.453$, $p < 0.001$, partial $\eta^2 = 0.617$: Figure 2C and 2D]. Furthermore, all groups had a reduction in the density of Ki67-ir cells

between one and two weeks of exposure to hormones or vehicle [estrone-treated ($p = 0.028$, Cohen's $d = 2.265$), estradiol-treated ($p < 0.001$, Cohen's $d = 8.756$) and vehicle-treated females ($p < 0.001$, Cohen's $d = 2.742$)]. There were also a significant interaction effect of region by exposure time [$F(2, 26) = 3.406$, $p = 0.049$, partial $\eta^2 = 0.208$] and main effects of hormone [$F(2, 26) = 4.061$, $p = 0.029$, partial $\eta^2 = 0.238$], week [$F(2, 26) = 70.034$, $p < 0.001$, partial $\eta^2 = 0.843$] and region [$F(1, 26) = 18.033$, $p < 0.001$, partial $\eta^2 = 0.410$]. There were no other significant main or interaction effects on the density of Ki67-ir cells (all p's > 0.608).

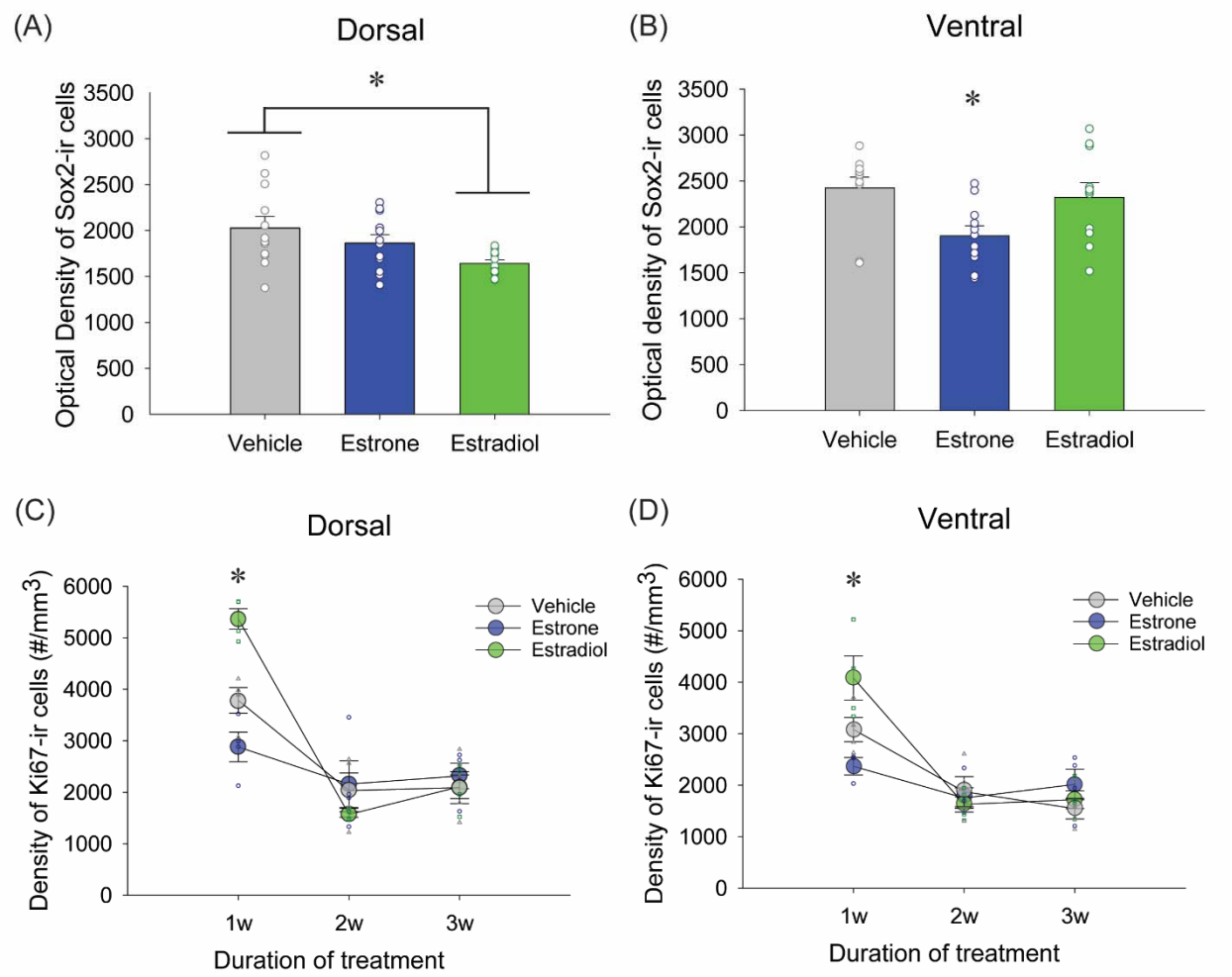

Fig. 2. A-B: Mean (±SEM) density of Sox2-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Vehicle-treated females had a greater density of Sox2-ir cells compared to estradiol-treated females in the dorsal dentate gyrus, and estradiol-treated and vehicle-treated females had a greater density of Sox2-ir cells in the ventral dentate gyrus. **C-D:** Mean (±SEM) density of Ki67-ir cells in the dorsal dentate gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females showed greater density of Ki67-ir cells compared to vehicle-treated females and compared to estrone-treated females, and vehicle-treated females showed greater density of Ki67-ir cells compared to estrone-treated females after one week of hormone exposure. * indicates $p < 0.05$. SEM-standard error of the mean.

3.3. Estrone and estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells compared to vehicle-treated females one week after cell division/ exposure to estrogens.

After one week of exposure to estrogens, estrone-treated ($p = 0.002$, Cohen's $d = 2.655$) and estradiol-treated females ($p = 0.004$, Cohen's $d = 1.868$) had a greater density of one-week old BrdU-ir cells compared to vehicle-treated females [interaction effect of week by hormone: $F(4, 24) = 3.120$, $p = 0.034$, partial $\eta^2 = 0.342$: Figure 3A], but there were no significant differences between treatment groups after two or three weeks in BrdU-ir cells (p's > 0.184). In both estrone and estradiol-treated females, there was a significantly greater density of BrdU-ir cells at one week compared to two weeks (estrone: $p = 0.004$, Cohen's $d = 3.955$; estradiol: $p = 0.001$, Cohen's $d = 2.980$) but no significant difference between two weeks and three weeks (p's > 0.118). In vehicle-treated females, there were no significant differences in the density of BrdU-ir cells between any of the weeks (p's > 0.302). There were also significant main effects of hormone [$F(2, 24) = 7.550$, $p = 0.003$, partial $\eta^2 = 0.342$], week [$F(2, 24) = 30.114$, $p < 0.001$, partial $\eta^2 = 0.715$] and region [$F(1, 24) = 4.284$, $p = 0.049$, partial $\eta^2 = 0.151$], but no other significant interaction effects on the density of BrdU-ir cells (p's > 0.288).

One week of exposure to estrogens also increased the density of BrdU/DCX-ir cells (estrone: $p < 0.001$, Cohen's $d = 2.529$; estradiol ($p = 0.001$, Cohen's $d = 1.903$) compared to vehicle-treated females [interaction effect of hormone by week: $F(4, 24) = 4.109$, $p = 0.011$, partial $\eta^2 = 0.406$: Figure 3B]. Treatment with estrogens decreased the density of BrdU/DCX-ir cells with each week of exposure [one to two weeks: p's < 0.001, Cohen's $d = 3.777$(estrone), Cohen's $d = 3.026$ (estradiol); two to three weeks: p's < 0.002, Cohen's $d = 8.906$ (estrone), Cohen's $d = 3.430$ (estradiol)]. This same pattern was not seen in vehicle-treated females (with no significant difference in density of BrdU/DCX-ir cells between one to two weeks ($p = 0.103$) but a decrease from two to three weeks of treatment ($p = 0.019$, Cohen's $d = 2.403$). There was

also a significant main effect of hormone [$F(2, 24) = 10.249$, $p < 0.001$, partial $\eta^2 = 0.461$] and week [$F(2, 24) = 101.756$, $p < 0.001$, partial $\eta^2 = 0.895$]. There were no other significant main or interaction effects on the density of BrdU/DCX-ir cells ($p > 0.167$).

In terms of the density of mature new neurons over the weeks, it was only the vehicle-treated groups that showed greater increase in the density of BrdU/NeuN-ir cells across time with an increase between one and three weeks ($p=0.018$, Cohen's $d = 3.974$) which was not seen in the groups treated with estrogens regardless of region ($p> 0.401$; interaction effect of hormone by week: $F(4, 24) = 2.963$, $p = 0.040$, partial $\eta^2 = 0.331$; Figure 3C). Furthermore, estradiol-treated and estrone-treated females had a trend for a greater density of BrdU/NeuN-ir cells compared to vehicle-treated females after one week (estradiol: $p = 0.089$, Cohen's $d = 3.225$; estrone: $p = 0.095$). There were also main effects of hormone [$F(2, 24) = 3.932$, $p = 0.033$, partial $\eta^2 = 0.247$] and week [$F(2, 24) = 4.963$, $p = 0.016$, partial $\eta^2 = 0.293$]. There were no other main or interaction effects on the density of BrdU/NeuN-ir cells (all p's > 0.208).

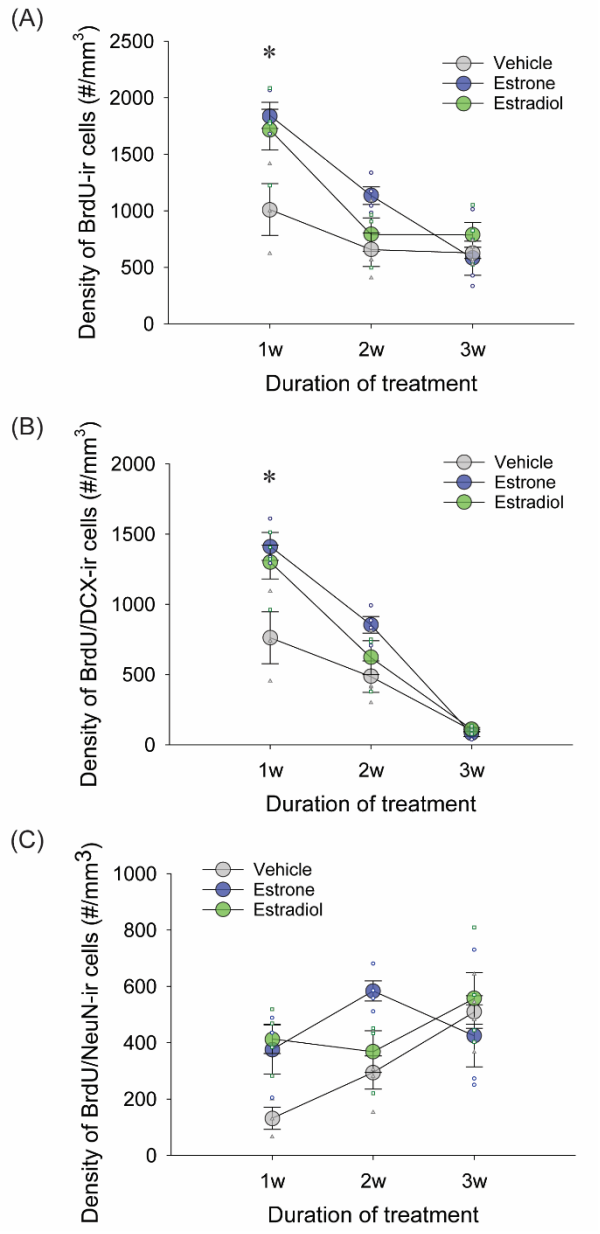

Fig. 3. A: Mean (±SEM) density of BrdU-ir cells in the dentate gyrus. **B:** Mean (±SEM) density of BrdU/DCX-ir cells in the dentate gyrus. Estrone or estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells in the ventral dentate gyrus compared to vehicle-treated females one week after BrdU injection and exposure to hormones. **C:** Mean (±SEM) density of BrdU/NeuN-ir cells in the dentate gyrus. Estradiol-treated females had a trend of greater density of BrdU/NeuN-ir cells compared to vehicle-treated females one week after BrdU injection and exposure to hormones. * indicates $p < 0.05$.

3.4. Estradiol-treated female rats showed greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after cell division and exposure to estrogens

Estradiol-treated females tended to have a greater percentage of BrdU/DCX-ir cells in the DG two weeks, compared to vehicle-treated females [$p = 0.067$, Cohen's $d = 4.139$; interaction effect of hormone by week: $F(4, 25) = 2.96$, $p = 0.040$; Figure 4A and 4B], but not after one week or three weeks of hormone treatment/after BrdU injection (p's > 0.188). Furthermore, the percentage of BrdU/DCX-ir cells was greater in the dorsal DG compared to the ventral DG after two weeks hormone exposure/ BrdU injection [interaction effect of week by region: $F(2, 25) = 5.04$, $p = 0.015$, partial $\eta^2 = 0.287$; post-hoc: $p = 0.011$, Cohen's $d = 1.249$]. There were main effects of week [$F(2, 25) = 2467.46$, $p < 0.001$, partial $\eta^2 = 0.995$] and region [$F(1, 25) = 4.62$, $p = 0.042$, partial $\eta^2 = 0.156$]. There were no other significant main or interaction effects on the percentage of BrdU/DCX-ir cells ($p > 0.306$).

As expected, the percentage of BrdU/NeuN-ir neurons increased with time such that a greater percentage of BrdU/NeuN-ir cells at three weeks compared to two weeks ($p < 0.001$, Cohen's $d = 2.159$), and a greater percentage of BrdU/NeuN-ir cells at two weeks compared to one week of estrogen exposure/BrdU injection ($p < 0.001$, Cohen's $d = 2.932$) [main effect of week: $F(2, 24) = 104.616$, $p < 0.001$, partial $\eta^2 = 0.897$: Figure 4C-D]. Estradiol-treated females, compared to vehicle-treated females, showed a lower percentage of BrdU/NeuN-ir cells in the dorsal DG after three weeks of treatment (a priori: $p = 0.0017$, Cohen's $d = 1.630$; interaction of region by hormone by week $F(2, 24) = 2.230$, $p = 0.096$, partial $\eta^2 = 0.271$). However, estradiol-treated females also had a higher percentage of BrdU/NeuN-ir at one week after hormone exposure/BrdU injection although this just failed to reach significance with Bonferroni corrections ($p=0.0045$). There was a trend for a significant main effect of region [$F(1, 24) = 3.136$, $p = 0.089$, partial $\eta^2 = 0.116$], but no other significant main or interaction effects on the

percentage of BrdU/NeuN-ir cells in the DG ($p > 0.202$).

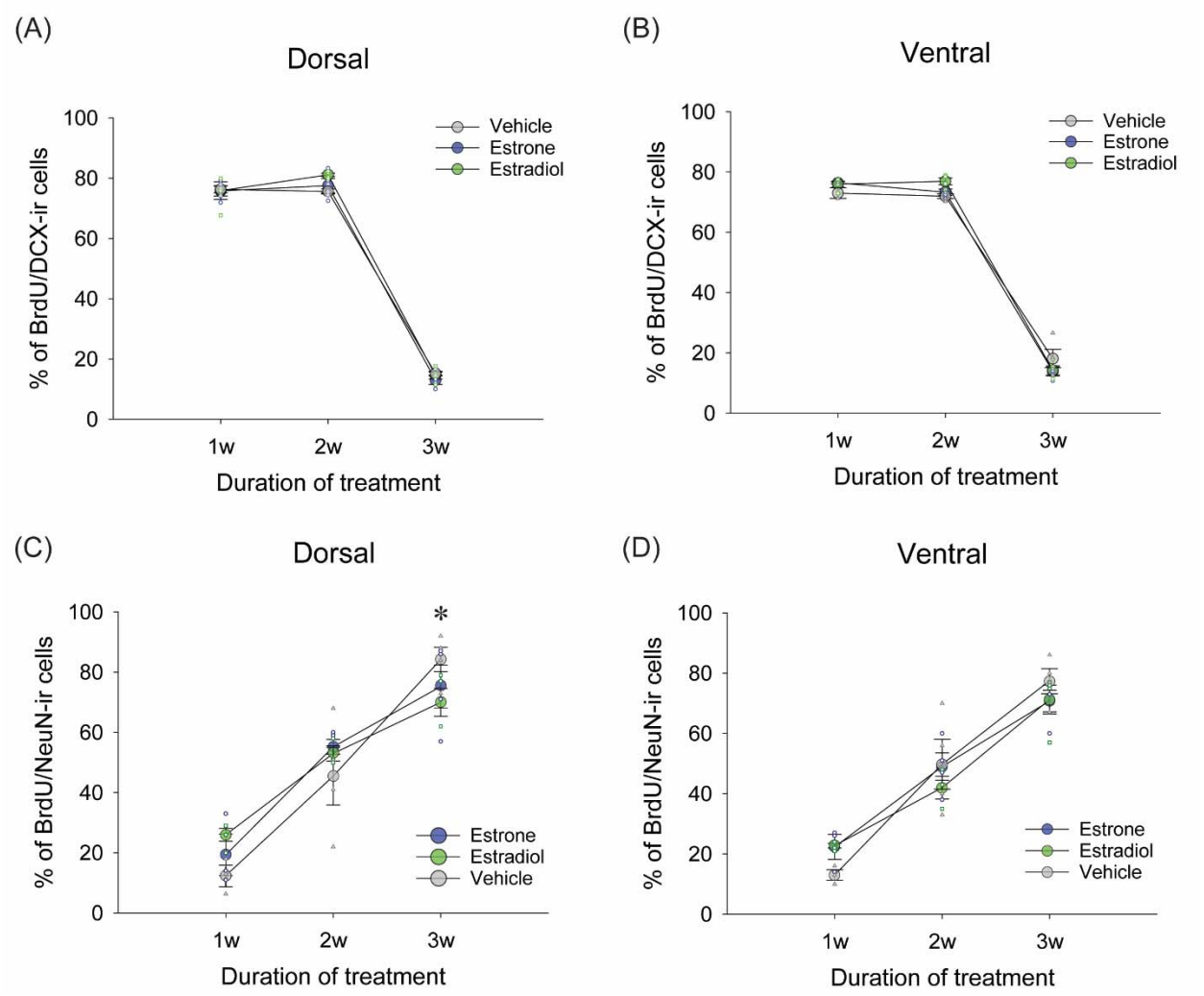

Fig. 4. A-B: Mean (±SEM) percentage of BrdU/DCX-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Estradiol-treated female rats showed a trend of greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after BrdU injection. **C-D:** Mean (±SEM) percentage of BrdU/NeuN-ir cells in the dorsal dentae gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females, compared to vehicle-treated females, showed a greater percentage of BrdU/NeuN-ir cells in the dorsal DG three weeks after BrdU injection/hormone exposure. * indicates $p < 0.05$.

4. Discussion

Both estrogens had a dynamic effect on different characteristics of neurogenesis that depended on duration of exposure to estrogens. Shorter exposure to estrogens (one week) increased the density of new neurons and enhanced early maturation of new neurons compared to vehicle exposure. However, longer duration of exposure to estrogens (2-3 weeks) resulted in greater attrition of immature neurons such that there was no longer a significant difference in the number of new neurons after three weeks of exposure to estrogens. These findings suggest that the pathways to neurogenesis differ with estrogens which may reflect early but not sustained neurogenic properties of estrogens. Perhaps surprisingly we found that estradiol decreased the density of neural stem cells in the dorsal DG, while estrone decreased the density of neural stem cells in the ventral DG compared to vehicle-treated ovariectomized rats. These findings highlight the importance of studying estrogen type and duration in females, which have important implications for treatments that promote hippocampal plasticity.

4.1. Estrone and estradiol reduce expression of neural stem cells in the DG.

The present study found that chronic administration of estradiol and estrone to ovariectomized rats reduced the density of neural stem cells in the DG. To our knowledge, this is the first study to report the effect of different estrogens on the density of neural stem cells. Interestingly, the effect of estrogens on neural stem cells varied along the dorsoventral axis, where estradiol reduced neural stem cells in the dorsal region and estrone in the ventral region. The dorsoventral axis of the hippocampus has differing functions where the dorsal region plays an important role in reference memory, and the ventral region plays an important role in stress, anxiety and working memory (reviewed in [33]). Previously, we found that intact females had a greater density of Sox2-ir cells in the ventral DG compared to dorsal DG [7] and this same

dorsoventral axis difference was seen in vehicle-treated and estradiol-treated females, but not in estrone-treated females. Underlying mechanisms or functional consequences for the differential regulation of estrogens on neural stem cells along the dorsoventral axis have yet to be determined.

4.2. Estradiol enhances, whereas estrone reduces, cell proliferation in the DG one week after treatment

Estradiol enhanced cell proliferation in the DG one week, but not after two or three weeks of hormone exposure. Interestingly, estrone had the opposite effect as it reduced cell proliferation after one week of exposure. These results suggest that estradiol, but not estrone, enhances cell proliferation in a limited time window (up to one week) in naïve rats. These results are consistent with previous work demonstrating that a single dose of estradiol enhances cell proliferation in the DG, whereas three weeks of repeated administration of estradiol had no significant effects on cell proliferation [10,12,15]. In contrast, the present study found that estrone decreased cell proliferation in the DG after one week of treatment. Previous work shows that a single dose of estrone treatment enhanced cell proliferation 30 minutes after administration [9], whereas three weeks of repeated administration of estrone had no significant effects on cell proliferation [14]. These findings indicate that chronic estrone treatment has detrimental effects on cell proliferation depending on the duration of treatment. Given the changes in cell proliferation with the different estrogens, but that the estrogens did not differ in neurogenesis levels after three weeks, this suggest that estradiol and estrone modulate neurogenesis via different pathways. Taken together, estrogens modulate proliferation in a type and duration-dependent manner.

4.3. Estrone and estradiol increase the density of one-week old neurons after one week of

exposure and increase the attrition of new neurons between one and two weeks.

Both estrogens given for one week increased the density of one-week old new dentate granule cells (DGCs; BrdU-ir cells) compared to vehicle treatment. However, by three weeks, there was no longer a significant effect of estrogens on the survival of new DGCs after three weeks of hormone treatment. Thus, the attrition of new DGCs was quite different between treatments with estrogens or vehicle as both estrogens, but not vehicle treatment, significantly reduced the density of new DGCs between one and two weeks. As majority of BrdU-ir cells (80%) at one and two weeks expressed an immature neuronal marker (DCX), the attrition of new DGCs were most likely due to the reduction of immature neurons. Indeed, the attrition of immature neurons (the density of BrdU/DCX-ir cells) between one and two weeks was also observed after treatment with both estrogens, but not after treatment with vehicle. It is possible that had we looked at an earlier time point than one week we would have seen a greater attrition of BrdU/DCX-ir cells as we saw the largest density of BrdU/DCX-ir cells 24 hours after BrdU injection in intact females [7]. In terms of mature new neurons (BrdU/NeuN-ir cells), only vehicle treatment increased the density across the weeks. These results indicate that although estrone and estradiol initially enhance adult neurogenesis (BrdU/DCX-ir and a trend for BrdU/NeuN-ir) at one week compared to vehicle treatment, due to significant attrition in these new neurons with exposure to estrogens across weeks, there is no pro-neurogenic effect of estrogens in a long term.

4.4. Estrogens' ability to increase maturation of new neurons is diminished after sustained exposure

In estradiol-treated rats, a slightly higher percentage of new DGCs expressed a mature neuronal marker (NeuN) at one week, whereas a lower percentage of DGCs expressed NeuN

three weeks after BrdU injection and hormone treatment compared to vehicle-treated rats. In terms of an immature neuronal marker (DCX), estradiol slightly increased the percentage of DGCs expressing DCX at two weeks after BrdU injection. These results suggest a possibility that estradiol initially enhances maturation of new neurons whereas prolonged exposure to estradiol delays maturation of new neurons in the adult DG, or that estrogens with time lose their effectiveness to enhance maturation of new neurons over time. Our previous work demonstrates that males show faster maturation of new neurons compared to females [7], and the present interpretation partially supports the hypothesis that estradiol contributes to the slower maturation time course of new neurons in females. Therefore, further research examining functional characteristics of these immature neurons is required to make a solid conclusion on the effects of estrogens on the maturation of new neurons.

4.5. Implications

Our results suggest that both the type of estrogens and duration of exposure to estrogens can significantly influence neurogenesis in the hippocampus. These findings are interesting as both animal and human studies suggest duration of exposure to estrogens influences a variety of factors. Estrogen exposure has differential effects on cell proliferation depending on time since ovariectomy surgery as estrogens enhance cell proliferation after short term (one week) ovarian hormone depletion [9–13], whereas estrogens do not significantly influence cell proliferation after long term (four weeks) depletion [12]. Here we found that one week of exposure to estradiol, but not longer exposure, enhanced both cell proliferation and maturation of new neurons after one week of ovarian hormone depletion. Our findings are reminiscent of findings from hormone therapy studies, as early initiation relative to menopause of hormone therapy increases hippocampal volume, whereas late treatment initiation relative to menopause has no

such beneficial effects on hippocampal volume [34]. In addition, we and others have found that the type of estrogens used can have opposing effects in humans and animal studies on neuroplasticity [14,21–25,35,36]. Here we found that one week of exposure to estradiol enhanced cell proliferation but one week of exposure to estrone decreased cell proliferation. This is consistent with findings that estradiol enhanced survival of new neurons after three weeks of exposure, whereas estrone decreased the survival, in rodents that underwent cognitive training in the Morris water maze [14]. Indeed, estradiol-based hormone therapy improves verbal memory and increase hippocampal volume [21,23,35], whereas conjugated estrone-based hormone therapy can have a detrimental effect in post-menopausal women [22,25,36]. Thus, in humans and in rodents, different estrogens modulate neuroplasticity and cognition depending not only on initiation of treatment relative to menopause/ovariectomy, but also on the type of hormone therapy [9,10,36,37,12–15,21,22,25,35].

5. Conclusion

Here we report that estrogens influence different facets of neurogenesis dependent on the type and duration of exposure to estrogens. Our findings add to the growing literature that estrone and estradiol have similar but not equivalent effects on neurogenesis. We also show that the duration of exposure to estrogens have dynamic effects on neurogenic parameters with proneurogenic effects within one week of exposure that are no longer evident with prolonged exposure to estrogens. Our findings shed a light on importance of studying short and long-term consequences of exogenous estrogens on adult neurogenesis.

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Statement of Ethics

All experiments were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and their suffering during all procedures.

Conflict of Interest

The authors declare that they have no conflicts of interest to declare.

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Author Contributions

SY: Conceptualization, Methodology, Data curation, Writing- Original draft preparation, Visualization. **YW**: Data collection. **LG**: Conceptualization, Methodology, Analysis, Writing- Reviewing and Editing, Supervision.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Fig. 1. A: Schematic illustration for the experimental timeline. **B-E**: Photomicrographs for (B) Sox2-ir cells (red) with DAPI (blue), (C) Ki67-ir cells (red) with DAPI (blue), (D) BrdU-ir cells (red) with DCX-ir cells (green) and (E) BrdU-ir cells (red) with NeuN-ir cells (green). Scale bars in (B) indicate 50 μm and scale bars in (C)-(E) indicate 20 μm. All photomicrographs were taken by Zeiss Axio Scan.Z1 with 20x (B) or 40x (C-E) objectives.

Fig. 2. A-B: Mean (±SEM) density of Sox2-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Vehicle-treated females had a greater density of Sox2-ir cells compared to estradiol-treated females in the dorsal dentate gyrus, and estradiol-treated and vehicle-treated females had a greater density of Sox2-ir cells in the ventral dentate gyrus. **C-D**: Mean (±SEM) density of Ki67-ir cells in the dorsal dentate gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females showed greater density of Ki67-ir cells compared to vehicle-treated females and compared to estrone-treated females, and vehicle-treated females showed greater density of Ki67-ir cells compared to estrone-treated females after one week of hormone exposure. * indicates $p < 0.05$. SEM-standard error of the mean.

Fig. 3. A: Mean (±SEM) density of BrdU-ir cells in the dentate gyrus. **B:** Mean (±SEM) density of BrdU/DCX-ir cells in the dentate gyrus. Estrone or estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells in the ventral dentate gyrus compared to vehicle-treated females one week after BrdU injection and exposure to hormones. **C:** Mean (±SEM) density of BrdU/NeuN-ir cells in the dentate gyrus. Estradiol-treated females had a trend of greater density of BrdU/NeuN-ir cells compared to vehicle-treated females one week after BrdU

injection and exposure to hormones. * indicates $p < 0.05$. SEM-standard error of the mean.

Fig. 4. A-B: Mean (±SEM) percentage of BrdU/DCX-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Estradiol-treated female rats showed a trend of greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after BrdU injection. **C-D**: Mean (±SEM) percentage of BrdU/NeuN-ir cells in the dorsal dentae gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females, compared to vehicle-treated females, showed a greater percentage of BrdU/NeuN-ir cells in the dorsal DG three weeks after BrdU injection/hormone exposure. * indicates $p < 0.05$. SEM-standard error of the mean.