17β-ESTRADIOL, BUT NOT ESTRONE, INCREASES HIPPOCAMPAL NEUROGENESIS AND ACTIVATION OF NEW GRANULE NEURONS IN RESPONSE TO SPATIAL MEMORY IN ADULT FEMALE RATS

by

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Abstract

Estrogens fluctuate across the lifespan in women, with circulating 17β-estradiol levels higher pre-menopause than estrone and circulating estrone levels higher postmenopause than 17β-estradiol. Estrone is a common component of hormone replacement therapies, but research shows that 17β-estradiol may have a greater positive impact on cognition. Previous studies show that acute estrone and 17β-estradiol impact hippocampus-dependent learning and cell proliferation in the dentate gyrus in a dose-dependent manner in adult female rats. The current study explores how chronic treatment with estrone and 17β-estradiol differentially influences spatial learning, hippocampal neurogenesis and activation of new neurons in response to spatial memory. Adult female rats received daily injections of vehicle (sesame oil), or a 10μg dose of either 17β-estradiol or estrone for 20 days. One day following the first hormone injection all rats were injected with the DNA synthesis marker, bromodeoxyuridine. On days 11-15 after BrdU injection rats were trained on a spatial reference version of the Morris water maze, and five days later (day 20 of estrogens treatment) were given a probe trial to assess memory retention. There were no significant differences between groups in acquisition or retention of Morris water maze. However, the 17β-estradiol group had significantly higher, while the estrone group had significantly lower, levels of neurogenesis in the dentate gyrus compared to controls. Furthermore, rats injected with 17β-estradiol showed significantly higher levels of activation of new neurons in response to spatial memory compared to controls. These results provide insight into how estrogens differentially influence the brain and behaviour, and may provide insight into the development of hormone replacement therapies for women.

Preface

A version of this paper has been submitted on May 30th, 2012 for publication.

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This manuscript was conceived of and planned by Robyn E. Stewart and Dr. Liisa Galea after discussions with Cindy K. Barha. Robyn E. Stewart and Cindy K. Barha carried out the experimental work. Robyn E. Stewart carried out the statistical analysis and writing of the thesis with the feedback and supervision of Dr. Liisa Galea.

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Table of Contents

List of Tables

List of Figures

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Recipe for a Masters Thesis:

1 supervisor perfectly prepared, with lots of flavour, and must be of the Galea variety

2 spoonful's of committee members

1 bunch of lab mates happy to help, with a readiness to teach, and a sense of humour

5 litres of friends (the supportive kind)

2 gallons of funding from NSERC and the Graduate Support Initiative at UBC

1 sprinkle of a beautiful city

8 ounces of parental and family support

1 pint of a future husband, Drew

When preparing, each ingredient is essential and adds a particular texture or flavour. Dr. Galea has been an incredible source of guidance and patience. Without her, this dish could easily become bitter. If the thesis is falling apart, it could be that you have to add more family and friends. Finally, without the bunch of lab mates, you can expect the thesis to take twice as long to cook.

Introduction

Estrogens fluctuate across the lifespan for women. There are three main forms of estrogens: estrone, estradiol and estriol. Estrone and estradiol shift dramatically during the transition from pre-menopause to post-menopause. Although both estrogens decline during perimenopause, there is a shift in the ratio between these two estrogens after menopause, such that while estradiol is found in greater amounts pre-menopause, estrone is more abundant post-menopause (Rannevik et al., 1995).

Hormone Replacement Therapy and Cognition

Hormone replacement therapy (HRT) has often been prescribed to peri- and post-menopausal women to combat the somatic and cognitive symptoms of menopause (Coope et al., 1975). Menopause is associated with an increased risk to develop cognitive decline and Alzheimer's disease (Henderson et al., 1994). Intriguingly the most prescribed HRT, Premarin, is comprised of a mixture of natural conjugated equine estrogens (CEE) but over 50% estrone (Kuhl, 2005). Considering that post-menopausal women have higher levels of estrone compared to estradiol, it seems counterintuitive that the most popular therapy uses estrone as the primary component. Studies have found conflicting results with relation to the effects of HRTs on cognition perhaps due to factors such as the timing of replacement, age of subject, and formulation of HRT with different therapies having different concentrations of estrogens (Hogervorst et al., 2000; Ryan et al., 2008). A large-scale study called the Women's Health Initiative Memory Study (WHIMS), compared effects of Premarin and Premarin plus progesterone on dementia and global cognitive functioning in postmenopausal women (Shumaker et al., 2003). Findings revealed that Premarin and progesterone treatments increased risk for dementia while Premarin alone led to a slight increase in risk for dementia and both hormone formulations led to an increased risk for mild cognitive impairment (Shumaker et al.,

2003; Rapp et al., 2003; Espeland et al., 2004). However, there were several shortcomings with the WHIMS study. First, many women had concurrent diseases, such as heart disease, diabetes, obesity, and hypertension, making it difficult to look at the consequences of HRTs separate from these confounding factors. Indeed Brinton (2008) has suggested the healthy cell bias hypothesis to potentially help explain the negative results found in the WHIMS and other studies using aged postmenopausal women. This hypothesis purports that exposing women to estrogens prior to the commencement of physical and neurological decline will lead to beneficial outcomes; however exposing women after decline as already commenced such as many years after menopause, leads to exacerbation of negative symptoms. Therefore the negative effects seen in women treated with Premarin in the WHIMS may be related to the unhealthy status due to the advanced age of the participants and their cells. Secondly, the WHIMS study used a continuous schedule of administration while the average HRT user in the United States is on a cyclic schedule of administration, a potentially crucial difference (Harman, 2010). Third, the average age of postmenopausal women enrolled in the WHIMS study was 65, whereas the average age of menopause in the United States is 51.8 years old (Randolph Jr et al., 2004). Therefore, the women in the WHIMS study were past the point of menopause by approximately 14 to 28 years. Indeed, evidence suggests that prolonged ovarian hormone deprivation reduces the benefits of 17β-estradiol replacement on the central nervous system in female rats (Smith et al., 2010). Animal research in older female rats show that cognitive improvements were seen when estradiol replacement commenced immediately post-ovariectomy but not when replacement commenced 5 months post-ovariectomy (Walf et al., 2009). Findings show that within 9-15 months post-ovariectomy in female rats it is still possible to enhance long term potentiation (LTP) with 17β-estradiol administration, however this benefit is no longer visible at 19 months post-ovariectomy. The increase in the ratio between NMDAR (N-Methyl-D-aspartic acid receptors) and AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), an

indicator of LTP induction, is also lost in the CA3 to CA1 synapses at 19 months post-ovariectomy but is still present at 9-15 months (Smith et al., 2010). This has led to the formulation of the critical period hypothesis (Gibbs and Gabor 2003; Gibbs 2010). Evidence has accumulated supporting this hypothesis, showing that estrogen's ability to positively influence cognitive functioning declines as the amount of time in a gonadal hormone deprived state increases (Gibbs 2000; Daniel et al., 2006). Finally, the WHIMS study investigated the effect of Premarin, formulated from CEEs, on cognition and dementia risk among other variables and there is evidence to suggest that HRTs without CEEs are more effective for promoting cognition. A meta-analysis by Ryan (2008) revealed that the HRTs showing more beneficial impacts on cognition were composed primarily of 17β-estradiol, as opposed to Premarin, which is primarily estrone. These studies suggest that more investigation into how estrogens impact cognition is needed.

Estrogens and Spatial Performance

Estrogens impact performance on cognitive tasks such as spatial performance. Early work provides support that high endogenous levels of estrogens are negatively correlated with spatial performance in the young adult female rodent (Frye, 1995; Galea et al., 1995) and women (Hampson, 1990). Furthermore low doses of 17β-estradiol led to better spatial working memory while high doses of 17β-estradiol led to poorer spatial working and reference memory in young adult female rats (Holmes et al., 2002; Daniel et al., 1997; Galea et al., 2001). These results suggest a dose-dependent relationship between the level of 17β-estradiol and spatial performance, with lower levels facilitating but high levels of 17β-estradiol impairing spatial performance. Studies suggest that 17β-estradiol works directly in the hippocampus to exert its effects on hippocampus-dependent learning and memory in female rodents (Sinopoli et al., 2006; Zhao et al., 2010). It has been demonstrated previously that continuous estradiol replacement

improves object placement task performance in older female rats (Walf et al., 2009) and Morris water maze performance in young adult female rats (Talboom et al., 2008) and that 17β-estradiol serum levels correlated with better Morris water maze performance (Talboom et al., 2008). Performance on these two tasks relies on the integrity of the hippocampus, suggesting that estradiol modulates hippocampal function. Furthermore there are a number of studies showing a link between menstrual cycle status or estrogens and cognition and activity in the hippocampus in primates (Hao et al., 2003), suggesting that the effects of estrogens are not limited to rodents.

Fewer studies have examined the influence of other estrogens, such as estrone, on cognition. Recently previous research from our lab found that while both 17β- and 17α-estradiol showed a dose-dependent facilitation of hippocampus-dependent contextual fear conditioning, estrone either had no significant effect or impaired contextual fear conditioning (Barha et al., 2010). Intriguingly, post-training hippocampal infusions of either 17β-estradiol or estrone led to improved retention on the T-maze footshock avoidance task, in which performance is not dependent on the integrity of the hippocampus (Farr et al., 2000). These findings collectively suggest that the effects of estrogens on cognition depend on dose, type of estrogens and cognitive task.

Estrogens also influence neuroplasticity in the hippocampus. In the adult female, 17β-estradiol influences apical spine density in the CA1 region of the hippocampus (MacLusky et al., 2005; Woolley et al., 1990) and alters neurogenesis in the hippocampus (Barker and Galea, 2008; Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003; Gould et al., 1999). Fewer studies have examined the influence of estrone on neuroplasticity in the hippocampus. Acute estrone treatment increases both the synaptic protein synaptophysin and cell proliferation (Barha et al., 2010; Barha et al., 2009) in the hippocampus, but to date no studies have examined the influence of chronic estrone treatment on neuroplasticity.

Adult Hippocampal Neurogenesis

Adult neurogenesis occurs in the dentate gyrus of the hippocampus of most mammalian species studied, including humans (Eriksson et al., 1998). Neurogenesis is comprised of the processes of cell proliferation, differentiation, migration and survival (Cameron and McKay, 2001; Snyder et al., 2009a; Epp et al., 2011a; Barker and Galea, 2008). Neurogenesis in the dentate gyrus of the hippocampus has been implicated in spatial performance in rodents (Epp et al., 2007; Snyder et al., 2005). In the dentate gyrus, young adult rats generate 9 000 cells each day or more than 250 000 per month (Cameron and McKay, 2001), suggesting a turnover of about 6% of cells per month. While the majority of new neurons die before reaching two weeks of age (Cameron et al., 1993), certain factors such as estradiol, learning and exercise can modulate the level of cell survival (Barker and Galea, 2008; Kordower et al., 2010; van Praag et al., 1999; Gould et al., 1999).

The time course of new neuronal development has been described in rats (Snyder et al., 2009a). During the first 24 hours, progenitor cells have divided and daughter cells then reside in the subgranular zone (Cameron and McKay, 2001). The daughter cells then undergo migration in the following 1-5 days and extend axons from 4-10 days post division (Hastings and Gould, 1999). At three weeks of age, the activation levels of new neurons peak in response to learning and memory (Snyder et al., 2009a).

The level of neurogenesis can be altered by either the number of cells being generated (cell proliferation) and/or the number of cells that survive (cell survival). The dentate gyrus is composed of the granule cell layer (GCL) and the hilus. The border between the GCL and the hilus is the subgranular zone. Within the subgranular layer reside progenitor cells that can divide, producing daughter cells (Cameron et al., 1993; Snyder et al., 2009a). Many of these daughter cells then migrate into the granule cell layer where they differentiate and take on morphology

akin to the mature cells that already reside there (Cameron et al., 1993; Ge et al, 2007). The mature cells have axons that synapse onto the pyramidal cells in the CA3 region of the hippocampus once they become function neurons (Ropireddy and Ascoli, 2011). Neurogenesis is being explored in this study as it relates to learning and memory.

Adult hippocampal neurogenesis is modulated by hippocampus-dependent learning and gonadal hormones, such as estradiol (Barha and Galea, 2010; Barha et al., 2010; Barker and Galea, 2008; Epp et al., 2011; Galea and McEwen, 1999; Holmes et al., 2002). For example, repeated estradiol treatment decreases the survival of new neurons in the dentate gyrus of the hippocampus in adult female, but not male, rodents (Barker and Galea, 2008). Hippocampal neurogenesis increases or decreases with exposure to hippocampus-dependent learning in male rats, dependent on task difficulty (Epp and Galea, 2009), quality of learning (Epp et al., 2007; Sisti et al., 2007) and age of new neurons at the time of exposure (Epp et al., 2011; Epp et al., 2007). To our knowledge, only two studies have investigated how neurogenesis might be impacted in females after training on a hippocampus-dependent task with equivocal results, likely depending on the type of task (Dalla et al., 2009; Chow et al., 2011), but no studies have investigated the role of estrogens to modulate this effect.

Activation in Response to Spatial Learning and Memory

There are several immediate early genes (IEGs) that respond to learning and memory including the immediate early gene product *zif268* that shows increased expression in response to the Morris water maze (Clark et al., 2012). An increase in IEG expression can be seen in response to neuronal activity. Specifically, zif268 can be upregulated by synaptic plasticity in the brain and is one of the most abundant proteins to do so (Li et al., 2005). It has been demonstrated that training on the Morris water maze can increase levels of activation in the dentate gyrus (Snyder et al., 2009b). Greater increases in activation were seen in young neurons compared to

older neurons already incorporated into the circuitry of the hippocampus. Importantly, differences in activation were not simply a function of experience in the maze. Increased activation was observed in new neurons in the Morris water maze group compared to both the cage controls and the swim controls. However, these differences in activation were not apparent in older neuronal populations (Snyder et al., 2009b). This suggests that activation of new neurons could be playing a vital role in the learning of the Morris water maze task as simply swimming in the environment is not sufficient to recruit the new neuron population. The same evidence can be used to negate the potential impact of stress on activation. As it is arguably more stressful for animals to be placed in the maze, the difference in activation levels between the Morris water maze group and the swim group suggests that this is an effect above and beyond a simple stress response. Recent research shows that new neurons in the hippocampus may preferentially respond to spatial compared to cued learning in male rodents (Kee et al, 2007; Epp et al., 2007). However, no studies have investigated activation (as assessed by immediate early genes) of new neurons in response to hippocampus-dependent learning after administration of 17β-estradiol or estrone.

The Current Experiment

The aim of this study was to explore how chronic high doses of estrone and 17β-estradiol impact hippocampus-dependent spatial reference memory, hippocampal neurogenesis and activation of new neurons in response to spatial memory. To examine this, rats were given daily injections of either a vehicle (sesame oil), or a 10μg dose of either 17β-estradiol or estrone for 20 days and trained on a spatial reference memory version of the Morris water maze. We hypothesized that chronic 17β-estradiol and estrone would differentially impact hippocampal neurogenesis and performance on the Morris water maze. We also hypothesized that chronic 17β-estradiol would significantly increase activation of new neurons, while estrone would

significantly decrease activation of new neurons in response to spatial memory compared to controls.

Experimental Procedures

Subjects

Subjects were 27 adult female Sprague-Dawley rats bred and raised at the University of British Columbia. The rats weighed between 208 and 260 g at the start of testing. All subjects were bilaterally ovariectomized through bilateral flank incisions while under anaesthesia using 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether (isofluorene, Boxter, Mississauga, ON, Canada). Rats were induced at a flow rate began at 5% and maintained at 2.5-3% to sustain a stable respiratory rate. Animals were also given 5mL of lactate ringer solution and 5mg/kg injection of a nonsteroidal anti-inflammatory analgesic (Anafen, MERIAL Canada Inc., Baie d'Urfe´, Quebec, Canada). The animals were left for a week undisturbed but monitored to recover from surgery. Subjects were housed individually in standard cages supplemented with a polyvinylchloride tube, paper towel, in addition to food and water *ad libitum*. Rats were housed on a 12:12 hour light/dark schedule with temperature maintained between 21° and 22° C. All the testing was conducted in accordance with the Canadian Council for Animal Care guidelines and was approved by the Animal Care Committee at the University of British Columbia. The minimum number of animals was used for this experiment and suffering was reduced as much as possible.

Apparatus

The Morris water maze task used in the current experiment consisted of a circular pool 180 cm in diameter. The pool was filled to 30 cm deep with 21^0C water and made opaque using non-toxic white paint (Reeves and Poole Group; Toronto, Ontario, Canada). There were multiple large visual cues on the walls around the room that remained stable throughout testing and a camera was positioned above the pool to track the movement of the rats in the pool. The camera

was connected to a computer and the program *ANY-maze* (Stoelting Co; Wood Dale, IL, USA) was used to record the trials. A 10 cm wide white platform was hidden 2cm below the surface of the water in the center of the northeast quadrant of the pool. The location of the hidden platform remained fixed throughout the duration of the experiment.

Procedure

Subjects were handled for five minutes per day over one week before the start of injections. Subjects were randomly divided into three groups to receive estrogens or vehicle (sesame oil). The groups were administered 17β-estradiol (n=9), estrone (n=9), or vehicle (n=9) starting on day 0 at a dose of 10μg. This dose for 17β-estradiol increases cell proliferation but reduces cell survival in the dentate gyrus (Barha et al., 2009; Barker and Galea, 2008; Tanapat et al., 2005; Tanapat et al., 1999). The same high dose was used for estrone, and increases cell proliferation and synaptophysin in the hippocampus (Barha et al., 2009, 2010). Animals received daily subcutaneous injections with a dose of 10μg of estrone or 17β-estradiol dissolved in 0.1 mL of sesame oil or 0.1 mL for the oil controls. All injections were given between 9:30 am and 11:30 am. On day 1, all 3 groups received a single intraperitoneal injection of bromodeoxyuridine (BrdU; 200 mg/kg). This was done so that high levels of estrogens were in circulation and influencing activity in the hippocampus when the DNA synthesis marker BrdU was given.

Beginning on day 12, rats were given five daily training sessions, each consisting of 4 trials per day/session. This time period for training relative to BrdU injection was chosen based on previous evidence that male rats, using the same BrdU-training timeline, had increased expression of activation (zif268) in new neurons after spatial, but not cued, training in the Morris water maze (Epp et al., 2011). Rats began training on the water maze approximately 30 minutes after hormone injection. Each trial lasted 60 seconds or the length of time that it took for the

animal to find the hidden platform. If the rat did not find the platform within 60 seconds, the experimenter guided it to the platform. The animal was left on the platform for 15 seconds before being retrieved and returned to their home cage. The inter-trail interval was 5 minutes. Within each session the individual trials would start from different pseudo-cardinal compass points and the same sequence would not be repeated across sessions, however each training group followed the same sequence of release points on each day. The hidden platform remained in the same location for each day and trial. Performance on this task is dependent on the integrity of the hippocampus (Morris, 1984). The computer program (*ANY-maze*) captured a number of variables including latency and distance to reach the hidden platform as well as percentage of time recorded in each quadrant and platform zone crossings during the probe trial. Performance was averaged for distance or latency to reach the hidden platform across each day of trials.

On day 20 post-BrdU injections, subjects completed a probe trial in which the hidden platform was removed. The probe trial lasted 60 seconds and was used to measure memory retention of the platform location. At 1.5 hours after the probe trial, subjects were administered an overdose of sodium pentobarbital followed by perfusion transcardially with 60ml of 0.1M phosphate buffered saline followed by 120ml of 4% paraformaldehyde. Blood was collected from the chest cavity at the time of perfusion. After 24 h, blood samples were centrifuged at 3000 rpm for 10 minutes and serum was retained for measurement of hormone concentrations through ELISA (Enzyme-linked immunosorbent assay) analysis. The brains were extracted and post-fixed using 4% paraformaldehyde for 24 hours before being transferred into 30% sucrose in 0.1M PBS. Using a microtome, brains were sliced throughout the hippocampus into 40μm sections and stored in 0.1M TBS (tris buffered saline). Sections were captured in series of every 10^{th} section.

ELISA Assays

In order to determine the circulating concentrations of estrone and estradiol in animals ELISA assays were conducted. This analysis was done as estrone and estradiol are interconverted through the enzyme 17b-hydroxysteriod dehydrogenases.

To quantitatively determine the concentration of estrone in circulation an ELISA was conducted on serum (GenWay Biotech, Inc. San Diego, CA, USA). The estrone conjugate was prepared by diluting estrone-biotin and avidin-HRP concentrates (1:100) into the assay buffer, mixing, and being allowed to stand for 15 minutes. Fifty microliters of the specimen were pipetted into each calibrator followed by 100µl of the conjugate solution, which was then incubated, on a plate shaker for one hour. The samples were washed three times with 300µl of diluted wash buffer and 150µl of TMB (tetramethylbensidine) was added. The plate was incubated on the shaker for 12 minutes after which 50µl of stopper solution (1M sulphuric acid) was added. Samples were then placed into an ELISA reader capable of reading absorbance at 450nm. The limit of sensitivity on the estrone kit is 10.0 pg/mL.

To determine estradiol concentrations in serum an ELISA was also conducted (GenWay Biotech, Inc. San Diego, CA, USA). 100µl of estradiol enzyme conjugate was added to 25µl of the samples in each well. The plate was placed on the shaker for 10-20 seconds and then allowed to incubate for 60 minutes at room temperature. The liquid was removed and the wells were washed three times with 300µl of buffer before adding 100µl of TIMB reagent and mixed for 10 seconds. The plate was allowed to incubate at room temperature for 30 minutes before 50µl of stop solution was added and mixed for 30 seconds. The plate was then read at 450nm with a microplate reader within 15 minutes. The limits of the detection with the estradiol kit are 3.94 pg/mL.

Immunohistochemistry

BrdU immunohistochemistry was performed on 40 μm sections as previously described (Epp and Galea, 2009). Briefly, the tissue was incubated in 0.6% H_2O_2 for 30 minutes followed by a rinse in 0.1 M TBS. The tissue was then place in 2N HCl for 30 minutes at 37° Celsius. A 0.1 M borate buffer was then used for 10 minutes at room temperature, subsequently rinsed three times in TBS and blocked by placing the tissue in TBS+ (96% TBS, 1% Triton X (10%), 3% Normal Horse Serum) for 30 minutes. The tissue was then incubated in 0.1% Triton X, 3% normal horse serum and 1:200 mouse anti-BrdU (Roche, Toronto, ON, Canada) for 48 hours at 4° C. The tissue was then rinsed three times in TBS followed by the secondary antibody, 1:200 anti-mouse IgG (Vector Laboratories, Burlington, ON, Canada) for four hours at room temperature. Afterwards, the tissue was rinsed three times in TBS and then incubated in ABC solution, prepared according to kit instructions (Vector), for 1.5 hours at room temperature. Slices were rinsed in the tissue three times in TBS and left at 4° C overnight. Finally, BrdU-ir cells were visualized with diaminobenzidine (DAB; Sigma, Oakville, ON, Canada). To finish, the tissue was mounted on glass slides, counterstained using cresyl violet and coverslipped with Permount (Fisher Scientific).

For double labelling of BrdU with NeuN a mature neuronal protein, slices were incubated in 0.1M PBS containing the primary antibody, 1:250 mouse anti-NeuN (EMD Millipore) at 4° C for 48 hours. The tissue was rinsed for 10 minutes three times in PBS. Tissue was then incubated in the secondary antibody, donkey anti-mouse Alexa 488 (1:200; Invitrogen Molecular Probes), for 18 hours. The tissue was then fixed using 4% paraformaldehyde for 10 minutes, rinsed two times for 10 minutes in saline, and were incubated in 2N hydrochloric acid at 37°C. Tissue was then incubated in rat anti-BrdU (1:500; AbD Serotec, Raleigh, NC, USA) for 48 hours at 4° Celsius, and then incubated for 24 hours in donkey anti-rat Cy3 (1:500; Invitrogen Molecular

Probes, Oregon, USA). The tissue was then rinsed three times for 10 minutes each in TBS and mounted glass slides using PVA DABCO, an anti-fading mounting medium, to cover slip.

BrdU and zif268, the immediate early gene product, immunoflorescence labelling began with three rinses for 10 minutes each of PBS. The tissue was then incubated for 24 hours in the zif268 primary antibody solution containing rabbit anti-zif268 (1:1000; Egr-1 SC-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 4% normal donkey serum, 0.03% Triton X, and PBS. The slices were then rinsed three times for 10 minutes in PBS. Tissue was then incubated in the secondary antibody donkey anti-rabbit Alexa 488 (1:500; Invitrogen Molecular Probes, Oregon, USA) for 18 hours at 4° Celsius. The tissue was then fixed in 4% paraformaldehyde for 10 minutes, washed twice in NaCl for 10 minutes each followed by 2N HCl at 37° Celsius for 30 minutes. Next there were three 10 minute rinses of PBS and then the tissue was incubated in mouse anti-BrdU (1:500; Roche Diagnostics GmbH, Mannheim, Germany) 4% normal donkey serum, 0.03% Triton X, and PBS for 24 hours at 4° Celsius. The tissue was then rinsed three times for 10 minutes after which it was incubated in donkey anti-mouse Cy3 (1:250: Jackson Immuno Research Laboratories Inc., Philadelphia, USA) for 18 hours. To conclude, the sections were rinsed three times for five minutes each and mounted on glass slides with cover slips using PVA DABCO.

Cell counting

BrdU-ir cells (see Figure 1A) were counted in every 10^{th} section throughout the granule layer and subgranular zone, as well as the hilus of the dentate gyrus. In order to approximate the total number of cells for each of the sections the total cell numbers were multiplied by 10 (Barker and Galea, 2008; Barha et al., 2009; Epp and Galea, 2009; Epp et al., 2011). The experimenter counting the cells was blind to the experimental group of the subject. Cells were counted in the hilus to account for potential changes in the blood-brain barrier permeability by

hormone treatment. Furthermore cells in the hilus are considered ectopic, and give rise to a different population of cells. Cells were counted using a Nikon E600 light microscope employing the 100x oil immersion objective. Areas were calculated separately for the hilus and the granule cell layer (with subgranular zone) using the software program ImageJ. Volumes were then determined by using the sum of the areas per animal followed by multiplying by the distance between sections (400μm) using Cavalieri's principle (Boyce et al., 2010).

The percentage of BrdU/NeuN -ir double-labelled cells were calculated by identifying 50 BrdU-ir cells per subject and verifying whether those cells were co-labeled with NeuN (see Figure 1E). The cells were assessed on an epifluorescent Nikon microscope with a 40x objective with the experimenter blind to the treatment groups. BrdU/zif268-ir cells were assessed in a similar manner with 100 BrdU-ir cells being identified and then examined for zif268 labelling (Fig 1C). For total levels of cell activation, approximations were acquired through the quantification of the immediate early gene product zif268 labelling in six sections (four dorsal, two ventral).

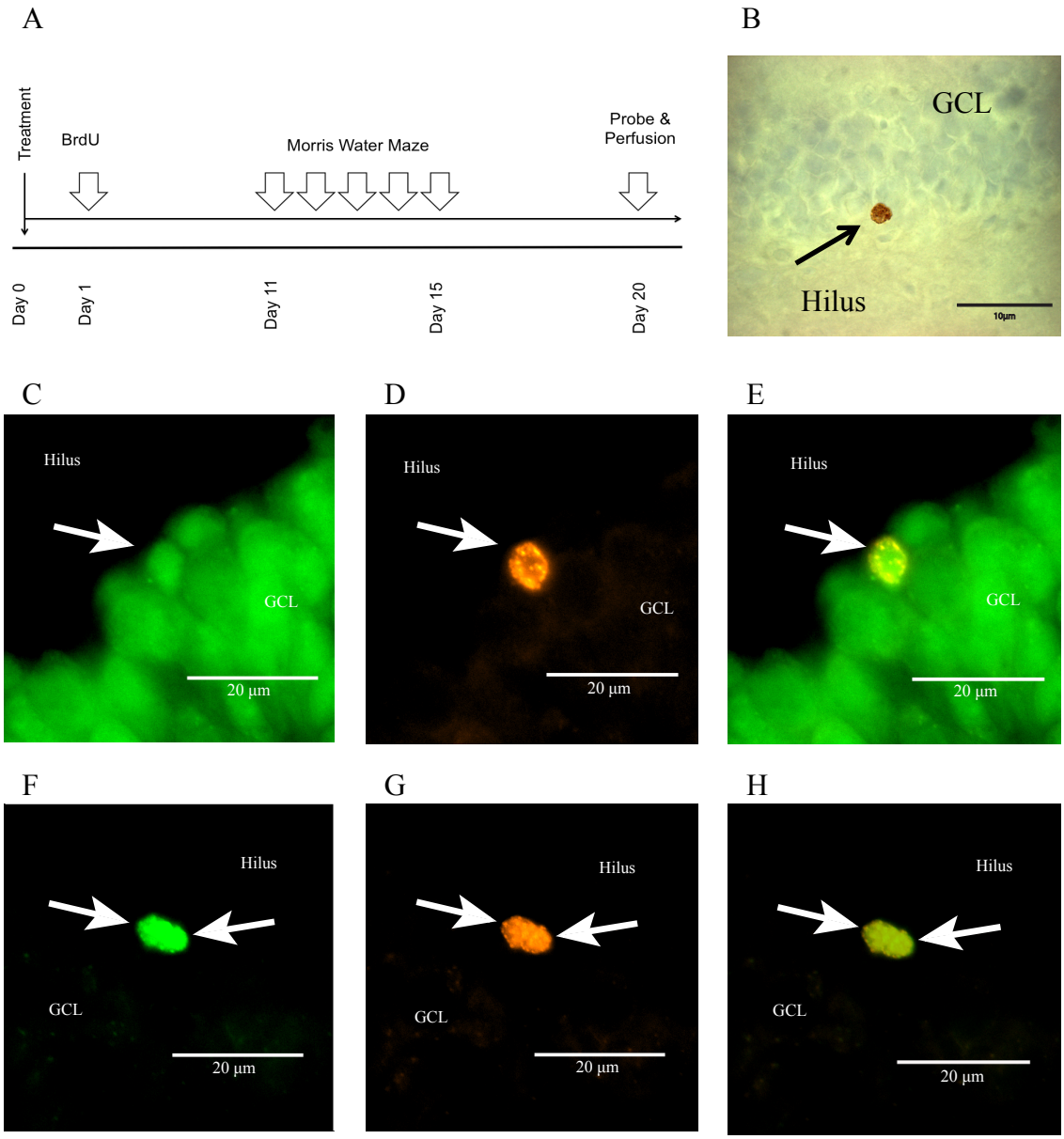


Figure 1A) Experimental outline B) photomicrographs of BrdU labeled in the GCL at 1000x C) Neuronal marker NeuN in the GCL at 600x D) BrdU labeled cell at 600x E) Merged images with an arrow indicating the double-labeled cells with BrdU (red) and NeuN (green) F) Zif268 labeled cells at 600x G) BrdU labeled cells at 600x H) Merged images with arrows indicating the double-labeled cells with BrdU (red) and zif268 (green).

Data Analyses

A repeated-measures analysis of variance (ANOVA) was conducted separately for the distance and latency to reach the hidden platform with day (1-5) as the within-subjects factor and treatment (17β-estradiol, estrone, vehicle) as the between-subjects factor. One-way ANOVAs were conducted on platform zone entries during the probe trial and the percentage of time spent in the target quadrant during the probe trial.

Separate repeated-measures ANOVA with treatment (17β- estradiol, estrone, vehicle) as the between-subjects factor and region as the within-subjects factor (granule cell layer, hilus) were calculated on the total number of BrdU-ir cells and the volume of the dentate gyrus. One-way ANOVAs were conducted on percentage of BrdU/NeuN-ir cells, total number of new neurons, and BrdU/zif268-ir cells with treatment as the between-subjects variable. Post-hoc analyses were conducted using Newman-Keuls comparisons. All analyses were completed using the software program Statistica (Statsoft Tulsa, OK). Two animals were removed from the brain analyses, one from the control group and the other from the 17β-estradiol group due to a lack of BrdU labelling. All statistical procedures were set at $\alpha = 0.05$ unless otherwise specified. A repeated measures ANOVA was conducted on the level of total activation with treatment (17β-estradiol, estrone, oil) as the between subjects factor and area (dorsal, ventral) as the within-subjects factor. Correlations were calculated between levels of new neuronal activation and 17β-estradiol, total BrdU-ir cells in the granule cell layer and total distance traveled across training days on the Morris water maze, total BrdU-ir cells in the hilus and percentage of time spent in the target quadrant during the probe trial, and estrogen levels (as measured by ELISA) versus total swim distance.

Results

Estrone and 17β-Estradiol Treatments did not Influence Performance in the Morris Water Maze Compared to Controls

There were significant main effects of day for distance ($F(617,12) = 50.07$, $p < 0.001$) and latency ($F(4,96) = 47.14$, $p < 0.001$) to reach the hidden platform, with distance and latency decreasing with increasing days, as expected (Figures 2A and2B). There were no other significant main or interaction effects between treatment and day for distance or latency to reach platform in the Morris water maze (all *p's* > 0.63). The probe trial also revealed no significant differences between treatment groups in the percentage of time spent in the target quadrant (p = 0.47; Figure 2C). There were no significant differences in platform zone entries ($F(2,22) = 0.40$, $p = 0.68$) between treatments.

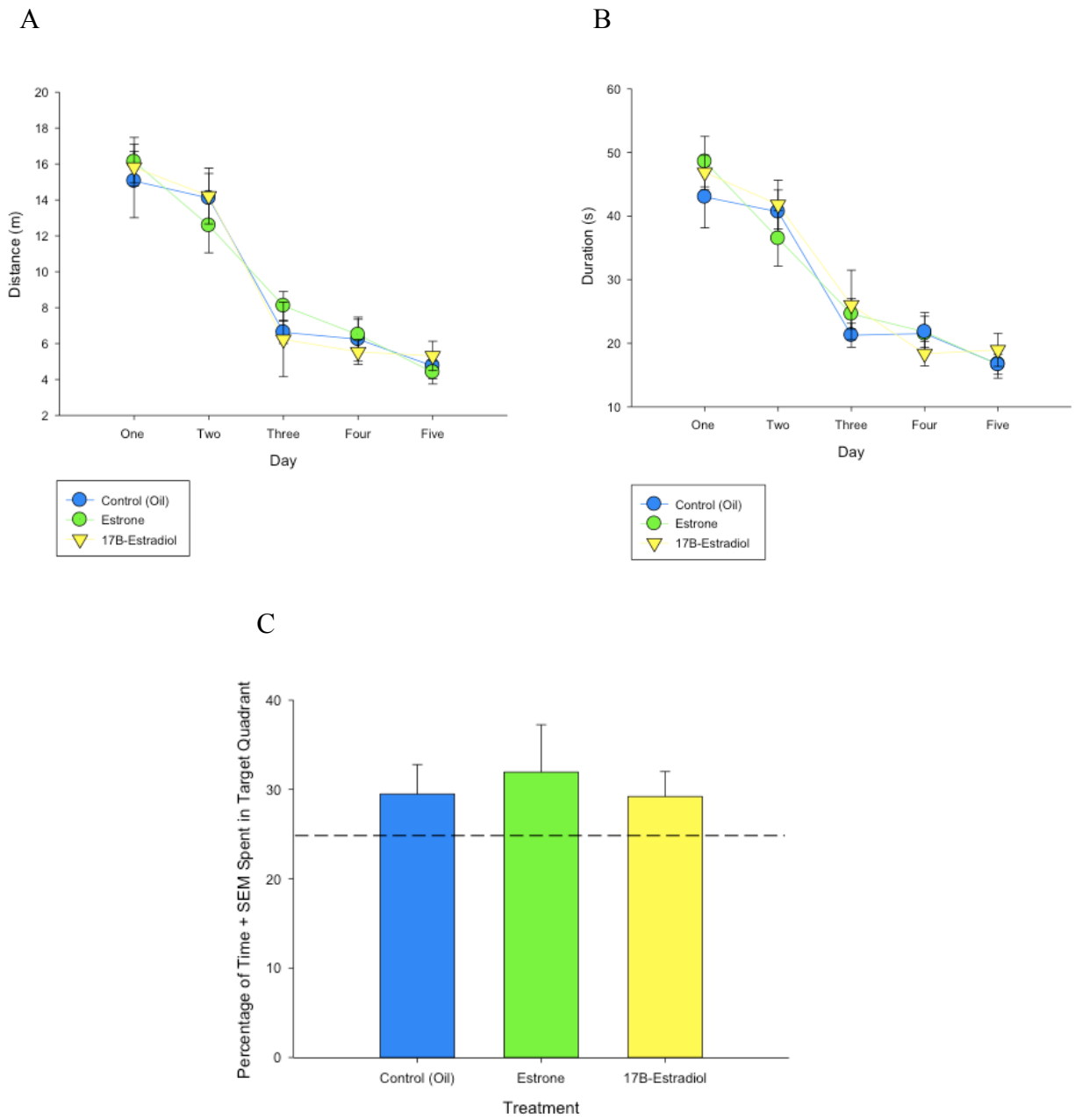


Figure 2A) Mean distance swum to reach the hidden platform (4 trials per day summed across 5 days). Data points represent mean distance and S.E.M. B) Mean duration to reach the platform per day. Data points represent mean duration and S.E.M. C) Percentage of time spent in the target quadrant during the probe trial and S.E.M.

There was No Significant Difference in Dentate Gyrus Volume Between Treatment Groups

There was no significant difference between treatment groups (see Table 1) in the volume of the granule cell layer ($p = 0.21$) or the hilus ($p = 0.12$), nor was there a significant interaction between treatment and region ($F(2,23) = 2.36$, $p = 0.12$). There was as expected a significant main effect of region ($p = 0.00015$), with the hilus having a larger volume than the granule cell layer. Because there was no difference in volume between the groups, the total number of BrdU-ir cells was used in all calculations.

Table 1. Mean values (± S.E.M.) of the granule cell layer (GCL) and hilus volume for treatments. There were no significant differences among treatment groups in GCL or Hilus volume. However, as expected the hilus was larger than the GCL for all groups.

Treatment	GCL Volume (mm^3)	Hilus Volume (mm^3)
Control (n=8)	2.34 ± 0.16	6.23 ± 0.40
Estrone (n=9)	2.07 ± 0.38	5.33 ± 0.41
17β-Estradiol (n=9)	2.00 ± 0.12	5.07 ± 0.35

Treatment with 17β-Estradiol Increased, while Treatment with Estrone Decreased, Hippocampal Neurogenesis Compared to Controls

There was a significant interaction ($F(2,22)=6.22$, $p = 0.007$) between treatment and region (see Figure 3A) and a significant main effect for treatment ($F(2,22)=6.01$, $p = 0.008$) and region ($F(1,22)=164.12$, $p < 0.0001$). 17β-estradiol significantly increased, while estrone significantly decreased, the total number of BrdU-ir cells in the granule cell layer compared with all other groups (controls vs 17β-estradiol $p = 0.046$; controls vs estrone $p = 0.005$; 17β-estradiol vs estrone $p = 0.0002$).

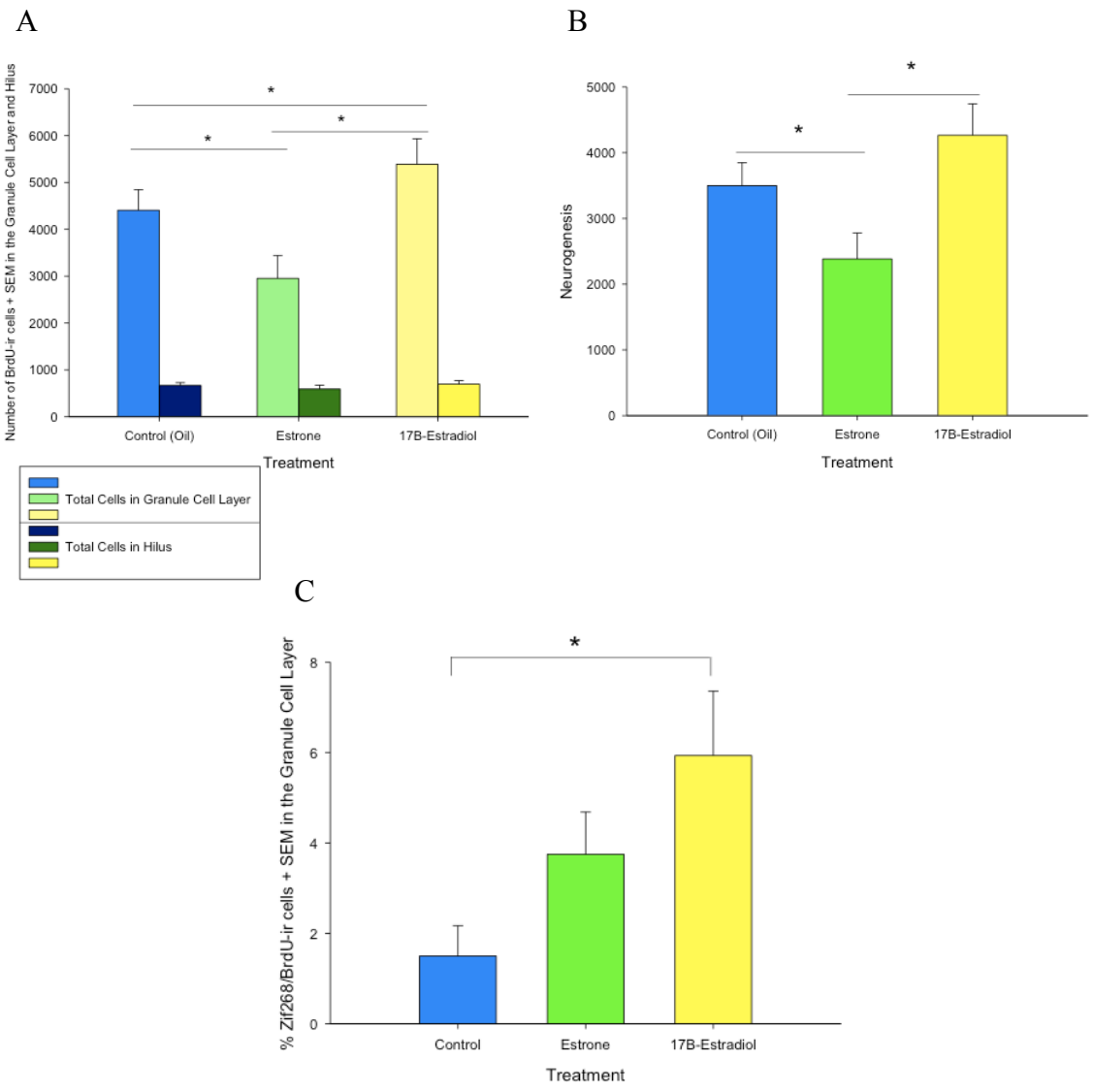


Figure 3A) Total BrdU-ir cells in the granule cell layer and hilus. Estradiol increased the number of BrdU-ir cells in the GCL while estrone decreased the number of BrdU-ir cells. Asterisks indicate significant difference between groups ($p < 0.05$). B) Levels of neurogenesis in the dentate gyrus of the hippocampus measured by multiplying of the number of BrdU-ir cells by the number of NeuN-ir cells. Asterisks indicate significantly different from the vehicle group. C) Mean percentage of BrdU/Zif268-ir cells was significantly higher in the 17β-estradiol treated group compared to controls. Asterisk indicates significantly different from the vehicle group ($p < 0.05$).

The percentage of BrdU-ir cells co-labelled with NeuN did not differ significantly between treatments ($p = 0.82$; Table 2). We then multiplied the total number of BrdU-ir cells with the percentage of BrdU-ir cells that were co-labelled with NeuN to get an approximation of neurogenesis. There was a significant interaction between treatment and region on neurogenesis ($F(2,22)= 5.50$, $p = 0.01$). Further analysis showed that estrone significantly decreased neurogenesis compared to both controls ($p = 0.009$) and 17β-estradiol ($p = 0.0003$), while 17β-estradiol significantly increased neurogenesis compared to estrone ($p = 0.0003$) and a strong trend compared to controls ($p = 0.06$)(Figure 3B).

Table 2. The percentage of BrdU-ir cells co-labelled with the mature neuronal marker NeuN across treatment groups. There was no significant difference in phenotype between treatment groups.

Treatment	% BrdU-ir co-labeled with NeuN
Control (n=8)	79.5 $\pm$ 1.40
Estrone (n=9)	81.6 $\pm$ 1.72
17β-Estradiol (n=8)	81.1 $\pm$ 3.34

Treatment with 17β-Estradiol Increased Activation of New Neurons (as Assessed by the Percentage of BrdU/zif268 Co-Labelled Cells) Compared to Controls

There was a significant main effect of treatment ($F(2,16) = 4.24$, $p = 0.033$) on the percentage of double-labelled BrdU/zif268-ir cells. As can be seen in Figure 3C, post-hoc analyses revealed a significantly higher percentage of BrdU/zif268-ir cells in the 17β-estradiol treated group compared with controls ($p = 0.022$) but no significant different between estrone and controls ($p= 0.15$) or 17β-estradiol versus estrone ($p = 0.16$).

We also examined total zif268 levels across the dorsal and ventral dentate gyrus in older granule neurons. There were no significant main or interaction effects between treatment groups in total zif268 expression in the granule cell layer of the dentate gyrus (all p's>0.18; Table 3).

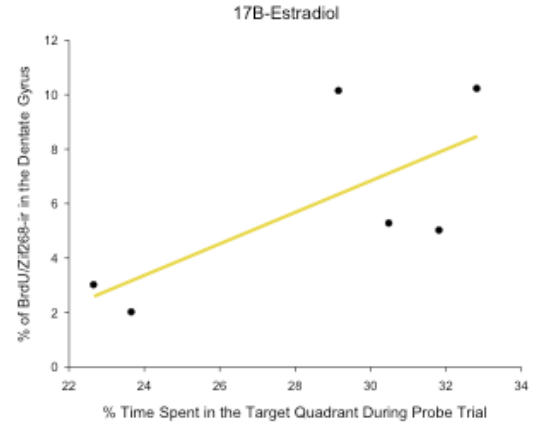
Table 3. Mean values (±S.E.M.) of total immediate early gene product, zif268, in the granule cell layer of the dentate gyrus for each treatment group. There was no significant difference in the total number of zif268-ir cells in the dorsal or ventral dentate gyrus between the treatment groups.

Treatment	Dorsal Slices	Ventral Slices	Total Activated Cells
Control (n=8)	54.88 ± 12.90	40.5 ± 7.37	95.38 ± 15.41
Estrone (n=9)	35.67 ± 3.80	53.44 ± 13.01	89.11 ± 14.92
17β-Estradiol (n=9)	41.11 ± 4.00	58.22 ± 11.87	99.33 ± 11.07

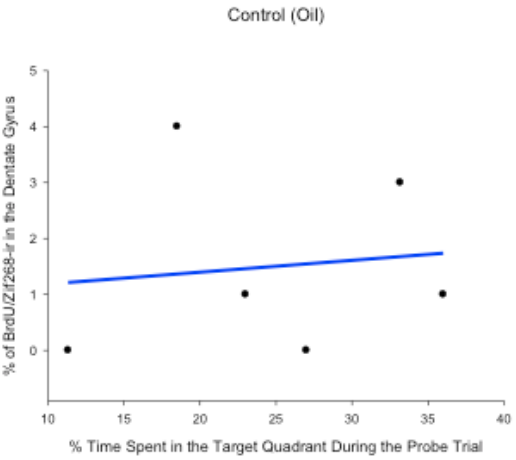
Correlations

Although not significant, a positive correlation was found between the percentage of time spent in the target quadrant during the probe trial and the level of BrdU/zif268-ir in the 17β-estradiol ($r(6)=0.71$, $p = 0.12$) (see Figure 4A). Thus increased activation of new cells was associated with spending more time in the target quadrant in the 17β-estradiol group. Intriguingly the same degree of positive correlation was not seen in either the estrone group ($r(7)=-0.05$, $p=0.92$) and the control group ($r(5)=-0.32$, $p=0.59$). Both groups had non-significant negative correlations indicating that increased activation, as shown through BrdU/zif268-ir cells was associated with less time spent in the target quadrant.

A)



B)



C)

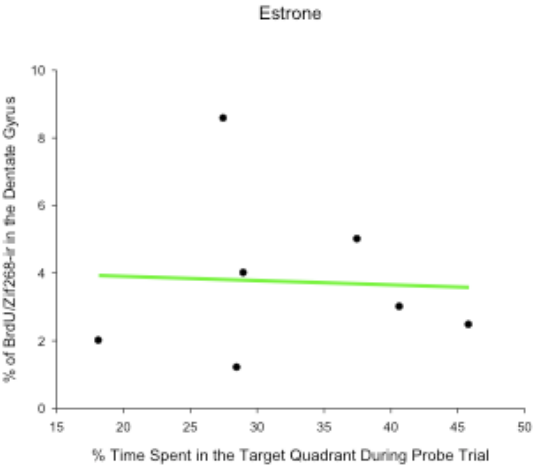


Figure 4A) Correlation between percentage of BrdU/Zif268-ir and percentage of time spent in the target quadrant during the probe trial for the 17β-estradiol group ($r(6)=0.71$, $p = 0.12$), B) control group ($r(5)=-0.32$, $p=0.59$), C) and the estrone group ($r(7)=-0.05$, $p=0.92$).

In addition, there was a significant positive correlation between total BrdU-ir cells in the granular cell layer and the total distance travelled across all trials for all treatment groups ($r(18) = 0.47$, $p = 0.045$)(see Figure 5), with more BrdU-ir cells surviving associated with worse total performance but this correlation was not significant when broken down by groups (all p's>0.18).

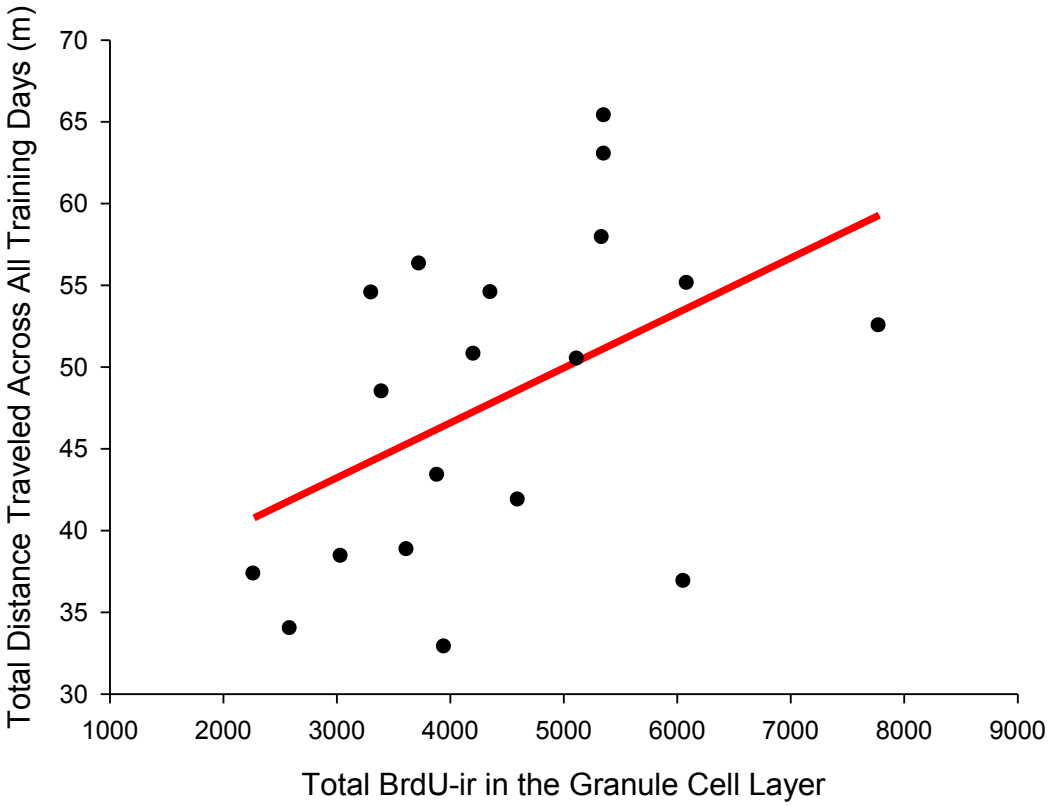


Figure 5) Correlation between the total distance traveled across all training days (metres) and total BrdU-ir in the granule cell layer across all groups ($r(18) = 0.47$, $p = 0.045$).

There was a significant negative correlation between BrdU-ir cells in the hilus and percentage time spent in the target quadrant in the control group only ($r(6) = -0.84$, $p = 0.037$) (see Figure 6), with more hilar cells (ectopic cells) being associated with worse performance.

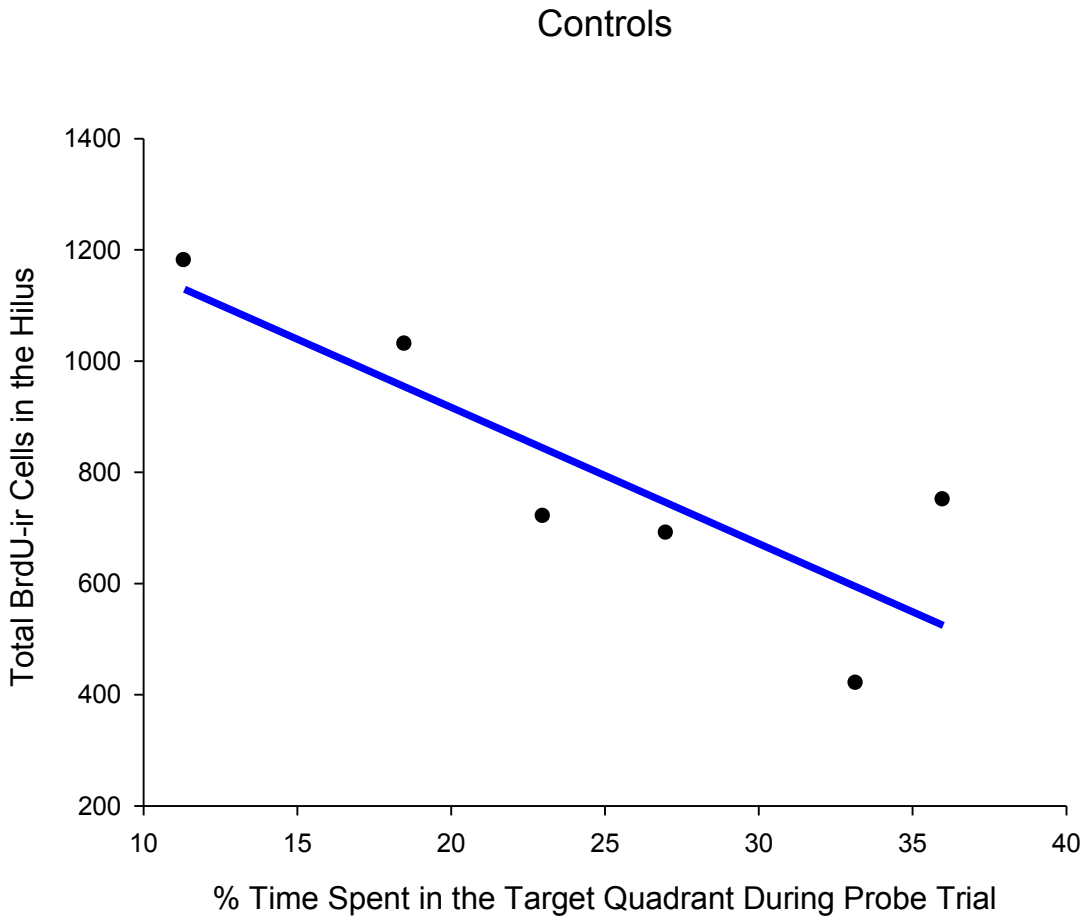


Figure 6) Correlation between the total BrdU-ir cells in the hilus and the percentage of time spent in the target quadrant during the probe trial only in the control group ($r(6) = -0.84$, $p = 0.037$).

17β-estradiol levels were significantly negatively correlated with total distance swum across all trials in the estrone group only ($r(7) = -0.84$, $p = 0.017$) (see Figure 7), indicating that higher 17β-estradiol levels were correlated with worse performance overall. There were no other significant correlations.

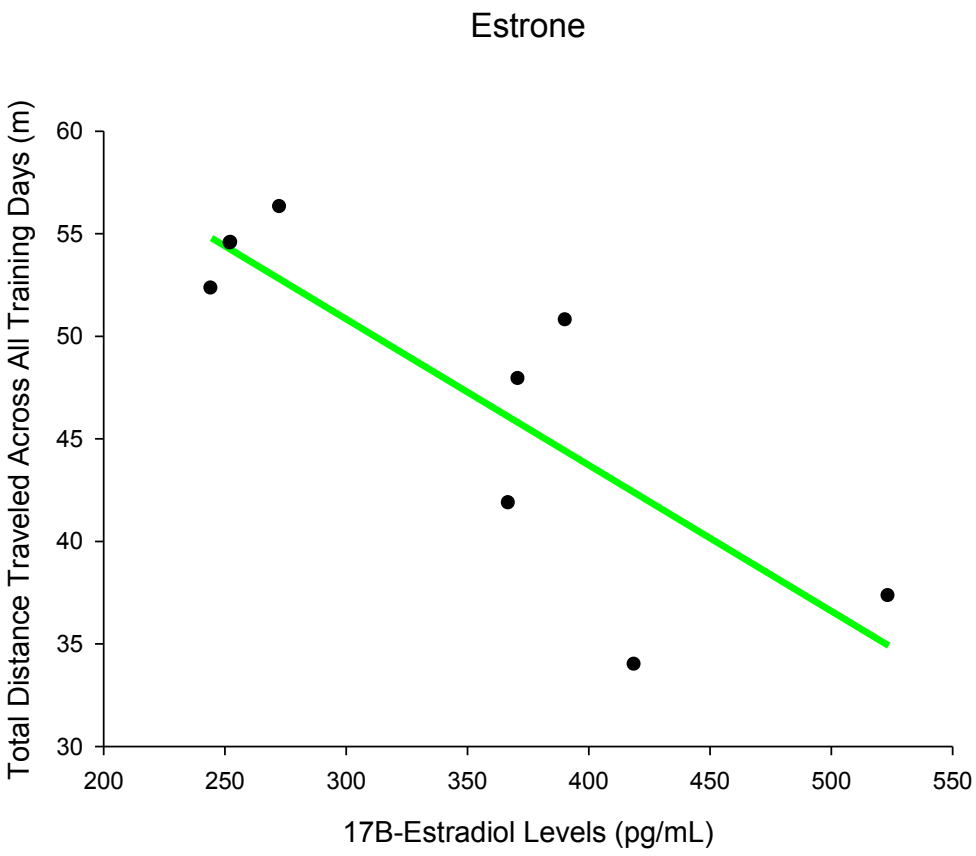


Figure 7) Correlation between the total distance traveled across all training days (metres) and the 17β-estradiol levels in the estrone group ($r(7) = -0.84$, $p = 0.017$).

Estrone and 17β-Estradiol Levels were Significantly Lower in the Control Group

The repeated measures ANOVA on serum hormone levels revealed a significant interaction of hormone type by treatment ($F(2,24)= 10.63$, $p < 0.001$) (Table 4). Further analysis showed control rats had lower levels of 17β-estradiol and estrone than both treatment groups (all $p's < 0.001$). There was no significant difference between 17β-estradiol and estrone ($p = 0.19$) in the control group. Concentration of 17β-estradiol was significantly higher in the 17β-estradiol group compared to the estrone group ($p = 0.05$). While there was only a trend for higher concentration of estrone in the estrone group compared to the 17β-estradiol group (0.07, one–tailed).

Table 4. Mean values (±S.E.M.) of the concentrations of estrone and 17β-estradiol found in the serum of treatment groups (pg/ml). Estrone and 17β-estradiol were significantly lower in the control group compared to the other groups. 17β-estradiol was significantly higher in the 17β-estradiol-treated group compared to all other groups. Estrone was significantly higher in the estrone-treated group compared to controls and tended to be higher compared to the 17β-estradiol group (p=0.07). Asterisks indicate significant differences.

Treatment	17β-Estradiol	Estrone
Control (n=9)	5.18 ± 0.63*	62.86 ± 5.71*
Estrone (n=9)	343.73 ± 31.85	271.69 ± 14.30*
17β-Estradiol (n=9)	408.69 ± 63.41*	185.32 ± 19.24

Discussion

The present results illustrate that repeated administration of the two estrogens, 17β-estradiol and estrone, differentially impact adult neurogenesis in the hippocampus without significantly affecting spatial performance in the Morris water maze at the 10 ug dose. Specifically, 17β-estradiol led to an increase in neurogenesis while estrone led to a decrease in neurogenesis in the hippocampus of adult female rats compared to controls in rats that had undergone spatial training. In addition, 17β-estradiol led to a significant increase in the percentage of new neurons expressing the immediate early gene, *zif268*, in response to spatial memory retrieval, while treatment with estrone failed to reach significance for percentage of BrdU/zif268-ir cells compared to controls. This study was the first to show that chronic administration of different estrogens can yield different results in relation to neurogenesis in the hippocampus and activation of these new neurons in response to spatial memory. Although these estrogens did not demonstrate any measurable effect on the behavioural learning and memory task, nor did they alter IEG response in the dentate gyrus of older cells, they did lead to differences in neurogenesis in the hippocampus and activation of those new cells in response to spatial memory retrieval. These findings add to the growing evidence that estrone and 17β-estradiol dramatically differ in their impact on neuroplasticity and provide a possible mechanism through which these two estrogens can vary in their effects on neuroprotection in women.

Treatment with High Levels of 17β-Estradiol and Estrone did not Significantly Impact Spatial Performance in the Morris Water Maze

In the present study we did not find evidence that a high dose of estrone and 17β-estradiol given prior to and during acquisition influence Morris water maze performance in female rats, which is partially inconsistent with previous literature (Kiss et al., 2012; Galea et al., 1995). These discrepancies between the current findings and those of past research could be due

to a variety of factors such as training parameters, the task chosen and/or the dose of estrogens chosen. A recent study showed improved performance in the Morris water maze task with the chronic administration of a lower dose of 17β-estradiol than used in this study; however, the animals also received approximately double the training (Kiss,et al., 2012). An acute 10ug dose of 17β-estradiol impaired hippocampus-dependent contextual fear conditioning (Barha et al., 2010) and the same dose of estradiol benzoate impaired spatial reference memory in the spatial working/reference memory version of the radial arm maze (Galea et al., 2001). However intra-hippocampal infusions of multiple doses of estradiol, facilitated object memory and consolidation when administered post-training (Gresack and Frick, 2006; Packard and Teather, 1997). It is important to note that these prior studies, while using tasks in which performance depends at least in part on the hippocampus, did not use the Morris water maze.

There are very few studies investigating the effects of estrone on learning. Consistent with the present study acute administration of the same dose of estrone resulted in no significant effect on contextual fear conditioning another hippocampus-dependent task that is also dependent on the amygdala (Barha et al., 2010). Other studies have examined the effects of Premarin, a conjugated equine estrogens, that is comprised of over 50% estrone sulphate and only 0.56% 17β-estradiol (Kuhl, 2005). Some studies find that cyclical treatment with Premarin (2 days on, 2 days off) enhances spatial performance (Acosta et al., 2009), while other studies find tonic treatment with Premarin impairs spatial preformance (Barha and Galea, under revision; Engler-Chiurazzi et al., 2012). In future it would be worthwhile to look at different chronic doses of estrone and estradiol as well as examining their impact on performance on different hippocampus-dependent tasks.

17β-Estradiol Increased Neurogenesis while Estrone Decreased Neurogenesis in the Dentate Gyrus Compared to Controls

In the present study we found that 21 days of 17β-estradiol treatment increased neurogenesis in rats trained on a spatial learning task, contrary to a finding where chronic estradiol benzoate decreased hippocampal neurogenesis in adult female rats (Barker and Galea, 2008). There could be several explanations for these conflicting results including concurrent spatial training, type of estradiol, the timing of estradiol administration in relation to BrdU administered. First, the rats in the current study were subjected to spatial training in the Morris water maze for 5 days out of their 21 days of hormone exposure. Spatial learning can increase or decrease hippocampal neurogenesis depending on a number of factors, such as task difficulty, quality of learning and age of new neurons at the time of exposure (Döbrössy et al., 2003; Gould et al., 1999; Epp et al., 2011; Epp et al., 2007). Intriguingly in male rats, spatial learning 11-15 days post BrdU administration followed by perfusion on day 20 led to decreased levels of neurogenesis (Epp et al., 2007). Thus, it is conceivable that the influence of estrogens on hippocampal neurogenesis is different when learning and memory is also involved. This is suggested by Frick et al. (2004) where increased spine densities in the CA1 region of the hippocampus were seen in the estradiol benzoate group compared to control but the effect disappeared when there was Morris water maze training. Furthermore in the present study we used 17β-estradiol while the Barker and Galea (2008) study used estradiol benzoate (benzoate allows estradiol to metabolise slower and may have contributed to these disparate findings). More importantly, in the current experiment, BrdU was injected 24 hours after the first dose of estrogens while in the Barker and Galea study BrdU was injected 24 h prior to injections with estradiol benzoate. This timing difference will result in dividing cells being labelled with BrdU under different hormonal milieus that can profoundly affect cell proliferation (Tanapat et al., 1999; Ormerod and Galea, 2001) and resulting neurogenesis. For example, estradiol and estrone

initially increases cell proliferation, and in the case of 17β-estradiol results in increased neurogenesis up to 14 days later (Tanapat et al., 1999; Ormerod and Galea, 2001; Barha et al., 2009). Thus the BrdU-ir cells in this study will have been labelled with estrogens (estrone and 17β-estradiol) on board while the Barker and Galea study investigated the effects of estradiol on cell survival independent of estradiol's effects on cell proliferation. Thus the increase in neurogenesis with 17β-estradiol in the present study would have been due to the initial increase in cell proliferation (Tanapat et al., 2005; Barha et al., 2009). Intriguingly, we know from our previous work that this same dose of estrone would have initially increased cell proliferation at the time that BrdU was administered in the present study, to approximately the same level as 17β-estradiol (Barha et al., 2009). Thus, given that we found that estrone decreased neurogenesis compared to all other groups, this suggests that chronic estrone decreased the survival of these new proliferating cells.

17β-Estradiol Increased the Activation of New Neurons in the Dentate Gyrus in Response to Spatial Memory Retrieval

In the present study we found that 17β-estradiol increased the activation of new neurons in the dentate gyrus in response to spatial memory retrieval. Thus new neurons produced and developed under the influence of 17β-estradiol showed a significantly higher level of activation, while the new neurons produced under the influence of high estrone were not significantly more activated than controls. Activation of new neurons (new neurons co-expressing the immediate early gene product zif268) shows that these new neurons are responsive to stimuli. In this case, the new neurons are activated in response to the retrieval of spatial memory. This is the first documentation of more activation of new neurons in the dentate gyrus created under 17β-estradiol in response to spatial memory. We also examined whether there were differences between groups on total activation (zif268) in response to the probe trial but did not find any

differences. This is partially inconsistent with previous research showing that acute high 17β-estradiol increased cfos-ir of all cells in the dentate gyrus of the hippocampus when using a spatial strategy on the Morris water maze (Pleil et al., 2011). The differences between the Pleil et al study and the present study is likely due to a number of factors: differences in IEGs (c-fos vs zif268), task, acute versus chronic 17β-estradiol exposure and/or timing. However, previous studies in mice have shown lower thresholds for LTP induction and enhanced synaptic plasticity in new neurons in the dentate gyrus (Ge et al., 2007). The more sensitive new neurons in mice have been compared with the 2-3 weeks time point in the development of new neurons in that adult rat hippocampus which coincides with the integration and maturation of the new neurons (Snyder et al., 2009a). The increased responsiveness of the new neurons is not maintained once the neurons mature (Ge et al., 2007) which could explain why activation is only impacted by estrogens in the new neurons in the present study. We found that neither estrone nor 17β-estradiol significantly influenced total zif268 expression in the dentate gyrus after spatial memory retrieval. To date, prior to the present study, there were no studies exploring whether estrone can influence IEG activation in the hippocampus in response to learning. The present study shows that there is a non-significant increase in IEG activation in new neurons in response to spatial memory in the dentate gyrus with estrone. Earlier investigations demonstrated that levels of activation of new neurons in response to spatial learning are at their highest three weeks post-BrdU in rats (Snyder et al., 2009a). Activation of cells, as measured by the immediate early gene *zif268* in the current experiment, suggests that these new cells are functional and influencing the hippocampal network. Previous work showed higher IEG activation during the probe trial after place training compared to cue training. Male rats trained with the hippocampus-dependent version of the Morris Water Maze had higher levels of zif268 and c-fos and Arc expression in new neurons than male rats trained with the hippocampus-independent version of the Morris Water Maze suggesting that retrieval of the spatial memory for the hidden platform

increased levels of activation of new neurons (Epp et al., 2011; Kee et al., 2007; Snyder et al., 2009a; Chow et al., 2011). Perhaps surprisingly, the increased activation (BrdU/zif68 expression) in the 17β-estradiol group seen here did not translate directly into significantly better performance on the probe trial. However, intriguingly there was a non-significant positive correlation between the level of activation in the 17β-estradiol group and the time spent in the target quadrant during the probe trial. The same correlation, activation and time spent in the target quadrant, was in the opposite direction for both the estrone and control groups. In other words, more activation with the IEG product zif268 led to better performance on the probe trial with the 17β-estradiol group but not in the estrone and control groups. This finding suggests that new neurons produced and developed under different hormone milieus react differently to appropriate stimuli in much the same fashion as new neurons produced with seizures or with running (Jakubs et al., 2006). In addition, it is also possible that examination of different brain areas will show differential activation with zif268 as an earlier study has suggested that activation in the hippocampus does not distinguish between different strategies used in an elevated T-maze however zif268 levels in the dorsal striatum that may more appropriately reflect behavioural differences in this task (Gill et al., 2007). Perhaps it would be worthwhile for future research to explore how activation and the employment of different learning strategies in other brain areas may be impacted in estrogen-enriched environments.

Zif286 was the IEG used in this study however it is possible that using a different IEG might yield a different picture (Davis et al., 2003). There are several IEGs that respond to learning and memory and zif268 has shown increased expression in response to the Morris Water Maze (Clark et al., 2012). Specifically, zif268 expression has been associated with memory retrieval (Hall et al., 2001). There are also IEGs responsive to long term potentiation (LTP), including zif268 and c-fos (Wisden et al., 1990; Richardson et al., 1992). One reason why

zif268 was chosen in the current study is the consistency with which the mRNA is increased in response to LTP. There is evidence to show that zif268 levels are related to the persistence of LTP (Richardson et al., 1992). It is possible, however, that another IEG would provide a different perspective on the memory formation process.

Conclusions

This study provides evidence that 17β-estradiol and estrone differentially impact hippocampal neurogenesis and activation of those new neurons in response to spatial memory. 17β-estradiol significantly increased cell survival, neurogenesis, and activation of new cells in response to spatial memory compared to controls. Intriguingly chronic estrone significantly decreased cell survival and neurogenesis in the dentate gyrus compared to all other groups. While there were no significant differences in the behavioural measures future research should explore different doses of estrogens and different hippocampus-dependent tasks. This is the first study to investigate the impact of repeated estrone on the Morris water maze and the use of chronic estrogens concurrent with the Morris water maze to investigate neurogenesis and activation of new neurons in the hippocampus. The effects of estrone on neuroplasticity and cognition remains understudied which is surprising considering the central role it plays in currently prescribed hormone replacement therapies. The present study is an important step in understanding more about how estrogens impact learning, memory, and neurogenesis in the dentate gyrus of the hippocampus.

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