Published in: Mahmoud R, Wainwright SR, Chaiton J, Lieblich SE, Galea LAM. (2016). Ovarian hormones impart resilience against chronic unpredictable stress and modulate the effects of fluoxetine on hippocampal plasticity in middle-aged female rats. *Neuropharmacology*, 107:278-293

Ovarian hormones, but not fluoxetine, impart resilience within a chronic unpredictable stress model in middle-aged female rats

Rand Mahmoud^{a,c}, Steven R. Wainwright^{a,c}, Jessica A. Chaiton^b, Stephanie E. Lieblich^b, and Liisa A.M. Galea^{b,c}

^a Graduate Program in Neuroscience, ^b Department of Psychology, ^c Centre for Brain Health, University of British Columbia, Vancouver Canada

Corresponding author:
Dr. Liisa Galea
Department of Psychology
University of British Columbia
2136 West Mall
Vancouver, BC
Canada, V6T 1Z4
Tel: +1 (604) 822 6536
Fax: +1 (604) 822 6923
Email: lgalea@psych.ubc.ca

Keywords: long-term ovariectomy, chronic unpredictable stress, depressive-like behavior, neurogenesis, antidepressant-efficacy

34

35

36

37 **Highlights**

- 38 • Long-term ovariectomy (OVX) increased depressive like-behavior in chronically stressed rats
- 39 • Long-term OVX altered basal and stress corticosterone levels in chronically stressed rats
- 40 • Fluoxetine increased neurogenesis and PSA-NCAM in the ventral hippocampus in an ovarian
- 41 status dependent manner
- 42 • Fluoxetine reduced microglial number in the ventral hippocampus of chronically stressed rats

43

44

45

46

47

48

49

50

51

52

53

54

ABSTRACT

Depression is more prevalent in women than in men, and women are at a heightened risk for depression during the postpartum and perimenopause. There is also evidence to suggest that the ovarian hormone milieu may dictate antidepressant efficacy. Thus, it is important to investigate the role of ovarian hormones in the pathogenesis of depression and in the mechanisms that may underlie antidepressant efficacy. In the present study, we used 10-month-old female Sprague-Dawley rats to examine the effects of long-term ovarian hormone deprivation on the development of depressive-like endophenotypes after chronic stress, and on antidepressant efficacy. Four months following ovariectomy (OVX) or sham surgery, all rats were subjected to 6 weeks of chronic unpredictable stress (CUS). During the last 3 weeks of CUS, rats received daily injections of fluoxetine (5 mg/kg) or vehicle. All rats were assessed on measures of anxiety- and depressive-like behavior, hypothalamic-pituitary-adrenal (HPA) negative feedback inhibition, and on markers of neurogenesis and microglia in the dentate gyrus. Our findings demonstrate that long-term ovarian hormone deprivation increased anxiety and depressive-like behavior, as seen by increased immobility in the forced swim test and latency to feed in the novelty suppressed feeding test, and decreased sucrose preference. Further, long-term OVX resulted in impaired HPA negative feedback inhibition, as seen in the dexamethasone suppression test. Fluoxetine treatment showed limited behavioral and neuroendocrine efficacy, however it reduced microglial (Iba-1) expression and increased cell proliferation, neurogenesis (via cell survival) and the expression of the polysialylated neuronal cell adhesion molecule (PSA-NCAM) in the dentate gyrus, and those effects varied by region (dorsal, ventral) and ovarian status. Taken together, our findings demonstrate that ovarian hormones may impart resilience against the behavioral and neuroendocrine consequences of chronic unpredictable stress, and may modulate the effects of fluoxetine on cell proliferation, neurogenesis, and PSA-NCAM in the middle-aged female.

1. INTRODUCTION

Women are more than twice as likely as men to develop depression (Gutiérrez-Lobos et al., 2002); a disparity that is particularly striking during the reproductive years of women (i.e. 25-50 years; (Gutiérrez-Lobos et al., 2002). Notably, periods that involve dramatic fluctuations and/or reductions in ovarian hormones, such as the postpartum and perimenopause, carry the highest risk of developing depression in women (Cohen et al., 2006; Hendrick et al., 1998; Soares, 2014). Together, these data provide compelling evidence for a role of ovarian hormones in the pathoetiology of depression.

Indeed, in healthy women reductions in ovarian hormones triggered sub-clinical depression scores, and estradiol levels were negatively associated with depressive symptoms (Frokjaer et al., 2015). In addition, withdrawal from a hormone simulated pregnancy in women with a history of postpartum depression increased depressive symptoms relative to women without a history of postpartum depression (Bloch *et al*, 2000). Thus, ovarian hormone fluctuations contribute to the development of depressive symptoms in women. Similarly in rats, estradiol withdrawal after hormone simulated pregnancy leads to depressive-like phenotypes (Galea et al., 2001; Green et al., 2009; Green and Galea, 2008). Importantly, in this model, treatment with estradiol or an estrogen receptor (ER) β agonist prevented the development of depressive-like behaviors (Galea et al., 2001; Green et al., 2009). Ovariectomy itself can increase depressive and anxiety-like behaviors while treatment with estradiol or selective estrogen receptor modulators (SERMs) can restore this effect (Bekku and Yoshimura, 2005; Li et al., 2014; Okada et al., 1997; Walf et al., 2004). Taken together, these findings corroborate the notion that reductions in ovarian hormones may increase the risk for developing a depressive-like endophenotype. However, most of these studies using females did not utilize a model of depression, and thus the face and construct validity of such studies is uncertain. The present

study aimed to fill this gap by investigating the role of ovarian hormones in the development of a depressive-like endophenotype within an animal model of depression.

Ovarian hormones are also implicated in antidepressant efficacy (Thase et al., 2005). For example, in postmenopausal women with depression, antidepressant treatment results in superior outcomes when prescribed alongside hormone therapy than when given alone (Thase et al., 2005), suggesting that ovarian hormones may enhance antidepressant efficacy. Furthermore, in women diagnosed with depression, estradiol has been given as either an adjunct therapy or a standalone antidepressant, with some success (Ahokas et al., 2001; Moses-Kolko et al., 2009; Rasgon et al., 2007). Similarly 17 β -estradiol treatment in ovariectomized rats augments the effects of the SSRI, sertraline, to reduce immobility in the forced swim test (Sell et al., 2008). However, this latter study was not conducted in conjunction with an animal model of depression, and thus it is unclear whether similar findings would be obtained from animals with a depressive-like phenotype. Our current study aimed to determine whether ovarian hormones influence antidepressant efficacy in an animal model of depression in middle-aged rodents.

The hippocampus exhibits compromised structural plasticity in depressed patients, as a meta-analysis showed that untreated depression is associated with reduced hippocampal volume, which is seen two years after diagnosis (McKinnon et al., 2009). Decreased hippocampal neurogenesis is observed in post-mortem tissue of depressed individuals (Boldrini et al., 2012) and in animal models of depression (Bessa et al., 2008; Green and Galea, 2008; Wainwright et al., 2011). Conversely, antidepressant treatment restores the reductions in neurogenesis in clinical depression and animal models (Bessa et al., 2008; Boldrini et al., 2012; Green and Galea, 2008). Notably, antidepressant use results in a greater increase in hippocampal volume in depressed women than depressed men (Vakili et al., 2000), and the pro-neurogenic effects of antidepressants are seen in the post-mortem tissue of women, but not men (Epp et al., 2013). Thus, ovarian hormones may modulate the effects of antidepressants on neurogenesis in the hippocampus.

There is also a growing recognition for the role of inflammation in depression and chronic stress (Miller et al., 2009). Remarkably, microglial activation is increased in the prefrontal cortex, insula and anterior cingulate cortex by approximately 30% in depressed patients relative to controls (Setiawan et al., 2015). Increased inflammation and microglial

activation in the hippocampus are also evident in animal models of chronic stress (Frank et al., 2007; Kreisel et al., 2013a). Despite the well-established anti-inflammatory effects of ovarian hormones in a variety of disease models (Habib and Beyer, 2015) for review), to our knowledge, the influence of ovarian hormones on microglia in relation to chronic stress or SSRI exposure has not been investigated, and thus the current study aimed to fill this gap.

Disturbances in the hypothalamic-pituitary-adrenal (HPA) axis are arguably the best characterized endocrine markers of depression. Specifically, a meta-analysis showed that depressed individuals display elevated levels of serum cortisol (Stetler and Miller, 2011), and at least a subset of depressed individuals show impairments in the HPA negative feedback system (Ising et al., 2007; Stetler and Miller, 2011). Antidepressant efficacy is more tightly linked to the restoration of HPA function in women compared to men (Binder et al., 2009) and hypogonadal women have impaired HPA negative feedback (Maes et al., 1992; Young et al., 1993). Therefore, similar to the effects of ovarian hormones on the behavioral efficacy of antidepressants, ovarian hormones may potentiate the efficacy of antidepressants to restore HPA dysfunction.

In this study, we investigated whether ovarian hormones impart resilience against the behavioral and HPA outcomes of chronic unpredictable stress in middle-aged female rats, and whether ovarian hormones influence antidepressant efficacy using fluoxetine under stress. Depressive-like phenotype was examined at the behavioral (forced swim test, novelty suppressed feeding test, and sucrose preference test), endocrine (cortisol levels and HPA negative feedback inhibition) and neural levels; examining both neuroplasticity (neurogenesis, PSA-NCAM), and microglial expression in the dentate gyrus. We hypothesized that ovarian hormones would be associated with resilience to the development of a depressive phenotype under chronic unpredictable stress, and that antidepressant efficacy would be modulated by ovarian hormone status in middle-aged female rats.

2. MATERIALS AND METHODS

2.1. Animals

Forty female Sprague-Dawley rats (Charles River Laboratories, Montreal, Canada), weighing 200–250g, were used in this study. Animals were pair-housed in a female-only colony room and

given *ad-libitum* access to food (Purina rat chow) and water. Colony rooms were temperature and humidity controlled ($21 \pm 1^\circ\text{C}$; $50 \pm 10\%$, respectively), and maintained on a 12-hour light/dark cycle (lights on at 07:00). All procedures were approved by the Animal Care Committee at the University of British Columbia, and performed in agreement with ethical guidelines set by the Canadian Council on Animal Care. All efforts were made to reduce the number of animals used and to minimize their suffering.

2.2. Surgery

Animals were randomly assigned to receive bilateral ovariectomy (OVX, $n=20$) or sham surgery (Sham, $n=20$), which were performed at approximately five months of age, as we have done previously (Barha et al., 2010). Ovarian hormone deprivation by ovariectomy was chosen as a model of surgical menopause (Barha and Galea, 2013). Surgeries were performed under isoflurane anesthesia, with ketamine (30mg/kg, Bimeda-MTC, Cambridge, ON), xylazine (2mg/kg, Bayer HealthCare, Toronto, ON) and bupivacaine (applied locally; 4mg/kg, Hospira Healthcare Corporation, Montreal, QC). Following recovery, animals were handled only during cage cleaning procedures, and left otherwise undisturbed until the beginning of the chronic unpredictable stress (CUS) procedure at approximately 9 months of age.

2.3. BrdU administration

To label dividing progenitor cells in the dentate gyrus and their progeny, all rats received two intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU; 200mg/kg; Sigma-Aldrich, St. Louis, MO, USA), given 8 hours apart, one day prior to the initiation of CUS. Two BrdU injections were chosen due to the lower rates of cell proliferation in the hippocampus in middle-aged rodents (Kuhn et al., 1996; Rao et al., 2005). Further, the timing of BrdU administration was chosen to delineate the effects of CUS (and subsequently antidepressant treatment) on the survival of newly produced cells, independent of treatment effects on cell proliferation.

2.4. Chronic Unpredictable Stress (CUS)

A CUS paradigm was used as we have done previously (Wainwright et al., 2011), with some modifications. Briefly, all animals were exposed to a variety of stressors, applied twice daily in a semi-random order at unpredictable times for a duration of 6 weeks. We allowed for at least 2 hours between the two daily stressors. The stressors and their descriptions are listed in Table 1.

2.5. Drug preparation and treatment

The first 3 weeks of CUS were utilized to develop a depressive-like phenotype. During the last 3 weeks of CUS (timeline depicted in Figure 1), all animals received daily subcutaneous injections of 5mg/kg Fluoxetine hydrochloride (Sequoia Research Products Ltd, Pangbourne, UK) or vehicle (Veh). Fluoxetine was dissolved in 5% DMSO (Sigma–Aldrich, St. Louis, MO, USA) in 0.9% saline, and given at a dose of 5mg/kg. Vehicle treatment consisted of 5% DMSO in 0.9% saline. Thus, we obtained four treatment groups (n=10 each), with drug and ovarian status as the between-subject variables: (i) vehicle-treated sham-operated rats (Veh-Sham); (ii) fluoxetine-treated sham-operated rats (FLX-Sham); (iii) vehicle-treated ovariectomized rats (Veh-OVX); and (iv) fluoxetine-treated ovariectomized rats (FLX-OVX). An intermediate dose of fluoxetine at 5mg/kg was chosen in order to avoid a possible ceiling effect, which may mask potential differences between ovarian status groups in the response to treatment. This rationale was based on prior work in ovariectomized middle-aged female rats showing that while 10mg/kg FLX decreased immobility in the forced swim test without hormone replacement, a low dose of 1.5mg/kg FLX decreased immobility only when administered in conjunction with estradiol replacement (Récamier-Carballo et al., 2012).

Figure 1. (A) Timeline depicting the sequence of experimental events. Sham surgery or bilateral ovariectomy (OVX) were performed at 5 months of age. Following a four month delay, rats were subjected to 6 weeks of chronic unpredictable stress (CUS; 24 hours after bromodeoxyuridine (BrdU) administration). Fluoxetine (FLX) or vehicle (Veh) treatment was administered daily for the final 3 weeks of CUS. Behavioral and neuroendocrine testing were initiated 24 hours after the final day of CUS, in the order shown (one test per day), with the exception of the sucrose preference test, which was administered weekly throughout CUS (not shown). Ninety minutes after the second session of FST, the animals were perfused and tissue was collected. D indicates day of experiment, beginning at BrdU administration on D0. NSF= novelty suppressed feeding, DEX= dexamethasone suppression test.

2.6. Behavioral testing

Behavioral testing occurred following the 6 weeks of CUS, with the exception of the sucrose preference test, which was administered weekly (see Figure 1).

2.6.1. Sucrose preference test. Rats were acclimatized to the 1% sucrose solution and the two-bottle procedure by introducing two identical bottles to their home cage for 24 hours, each containing 1% sucrose or tap water. After acclimatization, sucrose preference test was administered once prior to CUS to obtain a baseline measure of sucrose preference, and weekly thereafter until the termination of CUS (last test was on the last day of CUS). Briefly, the rats were singly-housed and simultaneously deprived of food and water for 4 hours, then presented with two bottles, one containing 1% sucrose and the other tap water. The test lasted 1 hour beginning at the start of the dark-phase (19:00-20:00hrs), after which the rats were re-paired with cage mates. All bottles were weighed before and after the test, and sucrose preference was calculated using the formula: $\text{sucrose preference} = (\text{sucrose consumed} / (\text{sucrose} + \text{water consumed})) \times 100$. The right-left placement of the sucrose and water bottles were counter-balanced for all animals between test days.

2.6.2. Novelty-Suppressed Feeding Test (NSF). The apparatus consisted of an open arena (60 x 60 x 50 cm) in which three pellets of a palatable food (Kellogg's Froot Loops) were placed in the center. Prior to the test, and to avoid neophobic reactions, the rats were acclimatized to the food by introducing four Froot Loops pieces to the home cage for three consecutive days. On test day, the food-deprived rats (18 hours) were individually placed in the arena, at a consistent corner of the chamber, and the latency to start feeding was measured. The rats were then immediately returned to the home cage, and the amount of Purina rat chow (in grams) consumed within the first hour was measured. The NSF arena was thoroughly cleaned with 70% EtOH between animals.

2.6.3. Forced Swim Test (FST). FST was conducted as we previously described (Wainwright et al., 2011). Briefly, each rat was subjected to two FST sessions in a vertical glass cylinder (45 x 28cm) filled with clean water (25 ± 0.5 °C) to a depth of 30 cm. Rats were individually placed in the cylinder for 15 minutes in the first session, and 5 minutes in the second session, which occurred 24 hours later. The test was videotaped and behavior was subsequently scored using the BEST Collection Software (Educational Consulting, Hobe Sound, FL, USA), by an observer blinded to treatment condition. Time spent in each of three distinct behaviors was scored: (1)

Immobility- floating with only those movements necessary to maintain the head above water; (2) Swimming- active paddling movements of forelimbs and/or hindlimbs; and (3) Struggling- vigorous climbing-like movements, with forelimbs surfacing above the water.

2.7. Blood sampling for basal corticosterone quantification

To assess changes in basal corticosterone levels throughout CUS, blood samples were collected every 10 days via the tail-vein, wherein the first sample was collected prior to the first stressor on day 1 of CUS. The procedure was consistently performed between 07:00-07:45hrs, and completed within 2 minutes of touching the home cage to avoid acute stress-induced increases in corticosterone. Blood samples were allowed to clot over night at 4°C, then centrifuged at 10 g for 15 minutes and serum aliquots were stored at -20°C until processing.

2.8. Dexamethasone Suppression Test

To evaluate the integrity of the glucocorticoid-dependent negative-feedback function of the HPA axis, the Dexamethasone suppression test (DEX) was administered 48 hours after the termination of CUS (see Figure 1). DEX was administered between 8:00-10:30hrs to all rats in order to avoid major circadian fluctuations in corticosterone. In short, 3 hours following the administration of Dexamethasone (in propylene glycol; i.p.; 100 ug/kg; Sigma) , blood was collected via the tail vein immediately before and after 30 minutes of restraint stress, and again 30 minutes after the termination of restraint (60 minutes after the first collection). Rats were left undisturbed in their home cages during recovery from restraint. Blood samples were allowed to clot over night at 4°C, then centrifuged at 10 g for 15 minutes and serum aliquots were stored at -20°C until processing.

2.9. Estrous cycle phase determination

In order to account for potential effects of estrous cycle phase on behavior and cell proliferation, vaginal cells were collected by lavage on experimental days 42-45 (Figure 1), estrous cycle phase was determined as previously described (Brummelte and Galea, 2010). Given the animals were in middle age at the beginning of CUS, the analysis of vaginal cells was also intended to determine if any rats in the sham group had reached persistent anestrous, however all rats were found to be cycling.

2.10. Tissue collection

Ninety minutes following session two of the FST, animals were deeply anesthetized with sodium pentobarbital and blood was collected via cardiac puncture. Animals were then transcardially perfused with cold 0.9% saline followed by 4% paraformaldehyde (PFA, Sigma–Aldrich) in 0.1M phosphate buffer (PB). Brains were extracted immediately and post-fixed in 4% PFA for 24 hours, then transferred to 30% sucrose solution in 0.1M PB (pH 7.4) and stored at 4 °C until sectioning. Brains were sliced in 40µm coronal sections using a Leica SM2000R microtome (Richmond Hill, ON, Canada), and were stored at -20 °C in a cryoprotective medium (0.1M PBS, 30% ethylene glycol and 20% glycerol; Sigma) until processing. Adrenals, thymus and ovaries (in sham-operated rats) were also extracted and weighed. Blood samples were allowed to clot over night at 4°C, then centrifuged at 10 g for 15 minutes and serum aliquots were stored at -20°C until processing.

2.11. Immunohistochemistry

All staining was performed on free-floating brain sections, and unless otherwise specified, incubation periods were conducted at room temperature and on a rotator.

2.11.1. BrdU. Sections were rinsed 3x10 minutes with 0.1M Tris-phosphate buffer (TBS; pH 7.4) between each of the following steps. After a 30 min incubation in 0.6% hydrogen peroxide (H₂O₂), sections were transferred to 2N HCL for 30 minute at 37 °C to denature DNA. Sections were then incubated in 0.1M Borate buffer for 10 min then blocked in TBS+ (containing 0.1% Triton X and 3% normal donkey serum (NDS)) for 30 minute. Sections were then transferred to the primary antibody solution (1:200 mouse monoclonal antibody against BrdU, Roche Diagnostics, Laval, Quebec, Canada) in TBS+ at 4 °C for 20 hours, and then the secondary antibody solution for 4 hours (1:200 anti-mouse IgG biotinylated, Vector Laboratories, Burlington, Ontario, Canada) in TBS+. Next, tissue was incubated in avidin-biotin complex for 1.5 hours (ABC; Elite kit; 1:50; Vector Laboratories). Immunoreactants were visualized by a peroxidase-diaminobenzidine (DAB) reaction (Vector Laboratories) for 7 minutes. Finally, sections were mounted on glass slides and allowed to dry, then counterstained with cresyl violet, dehydrated in increasing graded ethanol, defatted with xylenes, and cover-slipped with Permount.

2.11.2. Ki67. Sections were rinsed 3x10 minutes with 0.1M phosphate buffered saline (PBS; pH 7.4) between each of the following steps. After a 25 minutes incubation in 0.3% H₂O₂, sections

were transferred to a primary antibody solution for 16 hours, containing 1:1000 Rabbit anti-Ki67 (Vector Laboratories) monoclonal antibody solution in 0.1M PBS, 1% Normal Goat Serum (NGS, Vector Laboratories), and 0.5% Triton X. Afterwards, sections were incubated in a secondary antibody solution for 1 hour, containing 1:200 biotinylated Goat anti-rabbit IgG (Vector Laboratories, in 0.1M PBS), then in ABC (Vector Laboratories) for 40 min. Finally immunoreactivity was visualized with a 5 minutes DAB reaction (Vector Laboratories) and sections were mounted on glass slides and allowed to dry. Tissue was dehydrated in increasing graded ethanol, defatted with xylenes, and cover-slipped with Permount.

2.11.3. BrdU/NeuN. Sections were rinsed 3x10 minutes with 0.1M PBS (pH 7.4) between each of the following steps. Sections were incubated for 24 hours at 4 °C in a primary antibody solution containing 1:250 mouse anti-NeuN (Chemicon/Millipore), 3% Triton-X + 3% Normal donkey Serum (NDS) in 0.1M PBS. Tissue was then transferred to a secondary antibody solution for 18 hours at 4°C containing 1:200 donkey anti-mouse Alexa 488 (Invitrogen, Eugene, OR) in 0.1M PBS. Next, sections were incubated in 4% PFA for 10 minutes, then washed 2x10 minutes in 0.9% NaCl. To denature DNA, sections were subsequently incubated in 2N HCl at 37 °C for 30 minutes. Sections were then transferred to a primary antibody solution for 24 hours at 4°C, containing 1:500 Rat anti-BrdU (AbD Serotech, Oxford, UK) in 0.1M PBS + 3%NDS + 3% Triton-x. Next, sections were incubated in 1:500 Donkey anti-rat Cy3 (Jackson ImmunoResearch, Westgrove, PA) in 0.1M PBS for 24 hours at 4°C. Sections were mounted on glass slides and coverslipped with PVA-DABCO.

2.11.4. PSA-NCAM. Sections were rinsed 5x10 min with 0.1M TBS (pH 7.4) between each of the following steps. Sections were incubated in 0.3% H₂O₂ for 20 minutes, then transferred to a primary antibody solution at 4°C for 48 hours, containing 1:1000 mouse anti-PSA-NCAM (chemicon/Millipore) in 0.1M PBS + 0.3% Triton-X + 3% Normal horse Serum (NHS). Next, sections were incubated in a secondary antibody solution containing 1:250 horse anti-mouse IgG biotinylated (Vector) in 0.1M PBS for 24 hours at 4°C. Sections were then incubated in ABC for 2 hours, and immunoreactivity was visualized with a 5 minute DAB reaction (Vector Laboratories). Sections were finally mounted on glass slides and allowed to dry, then dehydrated in increasing graded ethanol, defatted with xylenes, and cover-slipped with Permount.

2.11.5. Iba-1. Sections were rinsed 3x10 minutes with 0.1M PBS (pH 7.4) between each of the following steps. Following a 25 minute incubation in 0.3% H₂O₂, sections were blocked for 1 hour with 10% normal goat serum (NGS) in 0.5% Triton X in 0.1 M PBS. Next, sections were incubated in a primary antibody solution for 18 hours, containing 1:1000 anti-Iba-1 (Wako, Osaka, Japan) in 10% NGS and 0.4% Triton X in 0.1M PBS. Afterwards, sections were transferred to a secondary antibody solution of 1:500 biotinylated anti-rabbit (Vector Laboratories) in 0.4% Triton-X and 2.5% NGS in PBS. Then, sections were incubated in ABC (Vector Laboratories) in 0.4% Triton-X in PBS for 1 hour, and immunoreactants were visualized by a Ni-DAB reaction (Vector Laboratories) for 7 minutes. Finally, sections were mounted on glass slides and allowed to dry, then counterstained with cresyl violet, dehydrated in increasing graded ethanol, defatted with xylenes, and cover-slipped with Permount.

2.12. Microscopy and cell counting

An experimenter blinded to treatment condition completed all immunohistochemical quantification under a Nikon E600 microscope equipped with epifluorescence. Representative photomicrographs are depicted in Figure 2. BrdU- and Ki67-immunoreactive (ir) cells were counted under 1000x magnification in every 10th section of the hippocampus along the rostral-caudal axis. To obtain an estimate of total immunoreactive cells in the dentate gyrus, raw numbers were multiplied by 10 (Hamson et al., 2013). BrdU- and Ki67-ir cells in the hilus were counted separately from those in the granule cell layer (GCL) and subgranular zone (SGZ, band approximately 50µm between the GCL and hilus). Counts were segregated in this way as cells in the hilus are considered ectopic and give rise to a distinct population of cells (Cameron et al., 1993). Furthermore, cells were quantified separately in the dorsal and ventral regions of the hippocampus, according to previously established coordinates (Banasr et al., 2004). The dorsal hippocampus is thought to be functionally distinct from the ventral hippocampus with the former being more important for spatial learning, and the latter being more important for stress and affect (Fanselow and Dong, 2010). To determine the proportion of BrdU-ir cells of neuronal phenotype, BrdU-ir cells in every 10th section of the hippocampus were examined for co-expression of NeuN under 400x magnification. An exhaustive count of PSA-NCAM and Iba-1-ir cells was completed in four hippocampal sections per animal, two sections each from the dorsal and ventral regions of the hippocampus (Kondratiuk et al., 2015), with approximately the following respective coordinates: (Bregma -3.12, -3.48, 6.00, and -6.36). PSA-NCAM-ir cells

were counted in the GCL only, whereas Iba-1-ir cells were counted (separately) in the GCL+SGZ and within an approximately 50µm band of the molecular layer (ML). Dentate gyrus areas (GCL and Hilus) were measured using digitized images of the sections and the software ImageJ (NIH).

2.13. Morphological analysis of Iba-1 immunoreactive cells

An experimenter blinded to treatment condition performed morphological analysis of Iba-1 immunoreactive cells in the GCL+SGZ. Specifically, using the measure feature of the NIS-Elements Basic Research software (Nikon) and under a Nikon E600 microscope, the number and length of processes were measured in 20 randomly selected cells from each animal, including 10 cells each in the dorsal and ventral regions of the hippocampus. The average length of processes was calculated for each cell, using the total length and number of processes for that cell, and subsequently an average value was taken for each animal, following previously established methods (Nemeth et al., 2014).

Figure 2. Representative photomicrographs of the granule cell layer (GCL) in the dentate gyrus, showing (A) BrdU-immunoreactive (ir; red), (B) NeuN-ir (green) (C) BrdU/NeuN-ir (merged image), (D) Ki67-ir, (E) BrdU-ir. (F) Iba-1 and (G) PSA-NCAM-ir cells. Images A-C, F-G viewed at 400x magnification, and D,E at 1000x magnification.

2.14. Determination of serum hormone levels

Radioimmunoassay kits were used according to the manufacturers' instructions to quantify serum corticosterone levels (Corticosterone, double-antibody RIA, MP Biomedicals, Solon, OH) and serum 17β -estradiol levels (Estradiol, Ultra-sensitive RIA, Beckman Coulter, Mississauga, Ontario). Samples were run in duplicates and the inter- and intra-assay coefficients were below 10%.

2.15. Statistical analyses

All statistical tests were performed using Statistica software (Tulsa, OK). Behavioral tests (Forced swim test, Novelty Suppressed Feeding, Sucrose Preference Test), serum 17β -estradiol levels and organ mass were each analyzed using factorial analysis of variance (ANOVA) with ovarian status (Sham, OVX) and drug treatment (Veh, FLX) as the between-subject factors. Percent sucrose preference and percent change in body mass were analyzed using a repeated-measures ANOVA with ovarian status (Sham, OVX) and drug treatment (Veh, FLX) as the between subjects variables, and weeks as the within-subject variable. Serum corticosterone (CORT) concentrations (basal or after DEX challenge) were each analyzed using a repeated-measures ANOVA with day (1, 10, 20, 30, 40) or time (0, 30, 60 min) as the within-subject variable, with ovarian status (Sham, OVX) and drug treatment (Veh, FLX) as the between-subjects variables. Ki67, BrdU-ir, Iba-1-ir and PSA-NCAM-ir cells, and the percentage of BrdU/NeuN colabeled cells were each analyzed using a repeated-measures ANOVA with ovarian status (Sham, OVX) and drug treatment (Veh, FLX) as the between-subjects variables and with hippocampal region (dorsal, ventral) and dentate gyrus area (GCL+SGZ, hilus) or (GCL+SGZ, ML) as the within-subject variables. A priori we expected differences between ovarian status groups on antidepressant efficacy. Post-hoc tests utilized Newman-Keul's comparisons and any a priori comparisons were subjected to Bonferroni correction ($\alpha=0.05$). Unless otherwise stated adding estrous cycle phase as a covariate did not significantly alter the results.

3. RESULTS

3.1. Ovariectomy increased immobility and decreased swimming and struggling behavior in the forced swim test

As predicted, OVX increased immobility in the FST (Figure 3A; $F(1, 36) = 15.998$, $p = 0.0003$, main effect of ovarian status). Interestingly, fluoxetine treatment did not significantly alter immobility in either group ($p = 0.20$), and there was no treatment by ovarian status interaction ($p=0.83$). Similarly, OVX decreased swimming and struggling in the FST (Figure 3B,C; $F(1, 36) = 10.401$, $p < 0.003$, $F(1, 36)=4.6799$, $p=.037$, respectively; main effect of ovarian status), but there were no other significant effects (all p 's > 0.2). When analyzing with estrous phase as a covariate, there was a significant behaviour by ovarian status interaction ($F(2,70)=3.71$,

p=0.029), in which OVX increased immobility ($p<0.0002$), decreased swimming ($p<0.003$), but had no significant effect on struggling ($p>0.3$).

3.2. Ovariectomy increased anxiety-like behavior in the novelty suppressed feeding test

As expected, OVX increased latency to feed in the NSF (Figure 3D; $F(1, 36)=18.502$, $p<0.0001$, main effect of ovarian status), but there were no other significant effects or interactions (all p 's >0.2). A priori comparisons revealed only a trend for fluoxetine to increase latency to feed in Sham rats ($p=0.09$). Importantly, the groups did not differ on food consumption in the hour following NSF (all p 's >0.1).

3.3. Ovariectomy increased anhedonia-like behavior in the sucrose preference test

Because drug treatment was initiated after 3 weeks of CUS, we ran a repeated measures ANOVA on sucrose preference with only weeks 4-6 of CUS as within subject variable. This revealed a significant CUS week by ovarian status interaction ($F(2, 72)=5.098$, $p=0.009$), in which as expected, OVX rats had a significantly lower sucrose preference on week 4 ($p<0.0004$; Figure 3E). Further, there was a significant CUS week by drug treatment interaction ($F(2, 72)=3.393$, $p=.039$), such that fluoxetine treatment significantly increased sucrose preference on week 6 of CUS ($p<0.025$) but not any other week (all p 's >0.4), regardless of ovarian status (Figure 3F). There were also significant main effects (ovarian status, and CUS week; all p 's <0.008), but no other significant interactions (all p 's >0.08). Importantly, the groups did not differ significantly on baseline sucrose preference prior to CUS (all p 's >0.05).

Figure 3. Effects of ovariectomy (OVX) and/or fluoxetine (FLX) treatment on behavior in the forced swim test (A-C), novelty suppressed feeding (D), and sucrose preference (E-F). In comparison to sham controls, OVX significantly increased immobility (A), and reduced swimming (B) and struggling (C) in the forced swim test, but fluoxetine treatment did not have a significant effect (A-C). (D) OVX significantly increased latency to feed in the novelty suppressed feeding test, and there was no significant effect of fluoxetine treatment ($p=0.09$ for FLX to increase latency to feed in Sham rats). (E-F) Effects of OVX and FLX treatment on preference for sucrose. OVX significantly decreased percentage of sucrose preference on week 4 of CUS, in comparison to sham controls (E). Fluoxetine significantly increased % sucrose preference on week 6 of CUS, relative to vehicle treatment (F). * Indicates $p < 0.05$, ** indicates

p <0.0004, and *** indicates p <0.0001 in relation to Sham controls. # Indicates p <0.025 in comparison to vehicle treatment. Veh= vehicle, FLX=fluoxetine. Data is represented in mean values + standard error of the mean (SEM).

3.4. Fluoxetine treatment significantly decreased body mass in OVX groups

Fluoxetine treatment significantly reduced percent change in body mass in OVX but not sham rats from week 4 of CUS onwards when compared to weeks 1-3 (all p's <0.03; week by drug treatment by ovarian status interaction: $F(5, 180)=2.723$, $p=0.021$; Figure 4A). Additionally, on week 6, the % change in body mass is significantly greater in fluoxetine-treated OVX rats when compared to vehicle-treated OVX rats ($p=0.047$). Furthermore, there were also significant main effects (ovarian status and CUS week, all p's < 0.01), and a CUS week by drug interaction ($p>0.00001$), but no other significant main effects or interactions (all p's >0.15).

3.5. Ovariectomy impaired glucocorticoid-dependent negative feedback on the HPA axis

OVX rats had significantly higher post-stress (DEX 30) CORT concentration compared to SHAM rats ($p <0.0001$; time by ovarian status interaction; Figure 4B, $F(2, 62)=7.63$, $p=0.0001$). Importantly however, there were no differences in serum CORT concentrations at baseline (DEX 0, $p=0.74$) or after recovery from stress (DEX 60, $p=0.94$). Fluoxetine reduced CORT levels at the 30 minute time point significantly only in Sham ($p=0.023$) but not OVX ($p=0.33$) rats. There was also a significant main effect of ovarian status ($p=0.005$), and a trend towards a drug treatment effect with fluoxetine attenuating CORT concentrations ($p=0.097$).

3.6. Sham rats showed an initial increase in percent change in basal CORT that was reduced by ovariectomy

OVX rats showed no significant percent change in baseline CORT across all weeks tested while the percent change was significantly lower than Sham groups across the first twenty days (Figure 4D, $F(3, 87)=3.83$, $p=0.013$, time by ovarian status interaction). In Sham rats, the percent change in CORT was significantly reduced by day 40 in comparison to days 10 and 20. There was also a significant main effect of time ($p=0.017$) and ovarian status ($p=0.0006$), but no other significant effects.

3.7. Ovariectomy reduced relative adrenal mass and modulated the effect of fluoxetine treatment on relative adrenal mass.

OVX significantly reduced relative adrenal mass (Figure 4C, $F(1,35)=5.90$, $p<0.02$, ovarian status by drug treatment interaction). Interestingly, post-hoc tests revealed only weak trends for fluoxetine to decrease relative adrenal mass in Sham rats ($p=0.096$) and to increase relative adrenal mass in OVX rats ($p=0.093$). There was also a significant main effect of ovarian status ($F(1,35)=61.53$, $p<0.0001$) but no other significant effects. Additionally, the groups did not differ on thymus/body mass (all p 's > 0.15).

3.8. Ovariectomy decreased serum 17β -estradiol levels

As expected, OVX significantly decreased serum estradiol in samples collected prior to perfusion (Figure 4D, $F(1,36)=18.88$, $p=0.0001$, main effect of ovarian status). There were no other significant effects (all p 's >0.4).

Figure 4. (A) Percent change in body mass across weeks of chronic unpredictable stress (CUS). Fluoxetine treatment significantly reduced body mass in ovariectomized (OVX) but not sham rats from week 4-6 of CUS when compared to weeks 1-3; * indicates $p < 0.03$. Fluoxetine treatment significantly decreased body mass in OVX rats on week 6, relative to vehicle treatment; # indicates $p < 0.05$. (B) Corticosterone (CORT) concentrations in the dexamethasone (DEX) suppression test. OVX rats had significantly higher post-stress CORT concentration relative to SHAM rats (DEX 30, $p = 0.000114$). Serum CORT concentrations at baseline (DEX 0),

or after recovery from stress (DEX 60) did not differ between groups. Fluoxetine significantly reduced post-stress CORT in sham ($p=0.023$) but not OVX ($p=0.33$) rats. **(C)** OVX significantly reduced adrenal/body mass ratio; * indicates $p<0.02$. **(D)** 17β -estradiol concentrations from serum taken on perfusion day. OVX significantly decreased serum 17β -estradiol relative to shams; * denotes $p=0.00011$. **(E)** Percent change in basal serum CORT was significantly lower in OVX groups compared to Shams across the first twenty days $p=0.013$. OVX=ovariectomy, Veh= vehicle, FLX= fluoxetine. Data is represented in mean values $\pm$ SEM.

3.9. Ovarian status and fluoxetine treatment did not significantly affect dentate gyrus volume

Apart from the expected volume differences between the GCL and hilus ($F(3, 102)=323.84$, $p<0.00001$, main effect of region), there were no significant differences between ovarian status or drug treatment groups (all p 's >0.3); thus the number of immunoreactive cells, instead of density, was used in all analyses.

3.10. Fluoxetine treatment increased the number of Ki67-ir cells in the GCL; dependent on hippocampal region and ovarian status

Fluoxetine treatment significantly increased the number of Ki67-ir cells in both the dorsal and ventral GCL+SGZ (p 's < 0.008) but not in the hilus (p 's >0.94 ; Figure 5A, $F(1, 36)=9.004$, $p<0.005$, region by area by treatment interaction). Furthermore, when compared to sham groups, OVX groups had a significantly higher number of Ki67-ir cells in the GCL+SGZ ($p<0.006$) but not in the hilus ($p=0.96$; area by ovarian status interaction $F(1, 36)=4.261$, $p=0.046$). A priori, we expected differences in the effects of fluoxetine treatment based on ovarian status, and comparisons revealed that when compared to vehicle, fluoxetine treatment significantly increased the number of Ki67-ir cells in both the dorsal and ventral GCL+SGZ in OVX rats (p 's <0.0002), but only in the ventral GCL+SGZ in Sham rats (ventral $p=0.00017$, dorsal $p=0.96$). In order to account for the effects of estrous cycle phase on cell proliferation (Tanapat et al., 1999), the number of Ki67-ir cells were analyzed with estrous phase as a covariate and did not change the main findings, furthermore there were no significant effects of the covariate (all p 's >0.78).

3.11. Fluoxetine increased the number of PSA-NCAM-ir cells in the ventral GCL in an ovarian status-dependent manner

There was a strong trend for fluoxetine treatment to increase the number of PSA-NCAM-ir cells in the ventral but not dorsal GCL+SGZ ($F(1, 33)=3.884$, $p=0.057$, region by treatment interaction; Figure 5B). A priori comparisons indicated that fluoxetine significantly increased

the number of PSA-NCAM-ir cells in the ventral GCL+SGZ of OVX animals ($p=0.0025$), but not in any other group or region (all $p's>0.1$). There was a significant main effect of region ($F(3,108)=101.16$, $p=0.0000$), a trend for a main effect of fluoxetine treatment ($p=0.084$) but no other significant effects (all $p's>0.3$).

3.12. Fluoxetine treatment increased the number of BrdU-ir cells in the dorsal GCL in an ovarian status-dependent manner

Interestingly, fluoxetine treatment significantly increased the number of BrdU-ir cells in the dorsal GCL of OVX rats (Figure 5C, $F(1, 34)=7.4185$, $p=0.01$, region by ovarian status by treatment interaction). As expected, there was a main effect of region and of area on BrdU-ir (all $p's<0.0001$). There were no other significant main effects or interactions (all $p's > 0.1$). Additionally, there were no group differences in the percentage of BrdU/NeuN colabeled cells, such that the average percentage of colabeled cells was above 75% for all groups (Table 2; all $p's> 0.75$).

3.13. Fluoxetine treatment decreased the number of Iba-1-immunoreactive cells in the ventral GCL, but had no effect on the average length of microglial processes

Fluoxetine treatment significantly decreased the number of Iba-1-ir cells in the GCL+SGZ ($p=0.0011$; Figure 5D) but not the ML ($p=0.55$; $F(1, 35)=4.56$, $p=.04$ area by treatment interaction), and there was a trend towards a main effect of drug treatment ($p<0.06$). Planned comparisons revealed that fluoxetine decreased the number of Iba-1-ir cells in the ventral ($p=0.000008$, $p=0.008$ in sham and OVX, respectively) but not dorsal GCL+SGZ ($p=0.016$, $p=0.39$, in sham and OVX, respectively). There were main effects of region and of area (all $p's<0.0001$), but no other significant effects (all $p's>0.1$). The average length of processes was significantly higher in the ventral relative to the dorsal GCL+SGZ, regardless of ovarian status (Table 3; $F(1, 35)=6.75$, $p=0.014$, main effect of region). There were no other significant main effects or interactions (all $p's>0.19$).

Figure 5. (A) Fluoxetine increased Ki67-ir in the dorsal and ventral granule cell layer (GCL) and subgranular zone (SGZ) in ovariectomized (OVX) rats, and in the ventral GCL+SGZ in sham rats, relative to vehicle treatment. (B) Fluoxetine increased PSA-NCAM-ir in the ventral GCL+SGZ in OVX rats, compared to vehicle treatment. (C) Fluoxetine increased BrdU-ir in the dorsal GCL+SGZ in OVX rats, compared to vehicle treatment. (D) Fluoxetine treatment significantly decreased Iba-1-ir in the ventral GCL, relative to vehicle treatment. Data is represented in mean values +SEM. Veh= vehicle, FLX= fluoxetine. * indicates $p < 0.01$, ** indicates $p < 0.003$, *** indicates $p < 0.0002$, and # indicates $p < 0.000001$, in relation to vehicle treatment.

3.14. Higher levels of estradiol are associated with reduced immobility in the forced swim test

Interestingly, serum estradiol was negatively correlated with percent immobility in FST in fluoxetine ($r = -0.6472$, $p = 0.043$; Figure 6A) but not vehicle treated rats ($r = 0.0982$, $p = 0.787$; Figure 6B).

Figure 6. Correlation of serum estradiol concentrations with percent time spent immobile in the forced swim test (FST) in Sham rats. Serum estradiol was negatively correlated with percent immobility in FST in fluoxetine treated rats ((A) $r = -0.6472$, $p = 0.043$) but not vehicle treated rats ((B) $r = 0.0982$, $p = 0.787$). If the outlier (serum estradiol=25.8 pg/ml) is removed from the analysis in (A), the correlation between serum estradiol and percent time spent immobile is strengthened ($r = -0.818$, $p = 0.007$).

4. DISCUSSION

Here, we report that under conditions of chronic stress, long-term ovarian hormone deprivation increased the vulnerability of middle-aged female rats to develop a depressive-like behavioral and endocrine phenotype. Interestingly, we found fluoxetine treatment showed weak behavioral and endocrine efficacy; fluoxetine treatment increased sucrose preference only in the last week of CUS and improved HPA negative feedback in Sham but not ovariectomized rats. Fluoxetine treatment was also associated with significant changes in neurogenesis, PSA-NCAM and microglia expression, in a region-specific and ovarian status-dependent manner. To our knowledge, this is the first report to integrate the behavioral, neurogenic, and neuroendocrine consequences of long-term ovarian hormone deprivation in tandem with chronic stress and antidepressant treatment. A schematic summary of findings is included in figure 7.

4.1. Ovarian hormones imparted resilience against the development of anxiety- and depressive-like behaviors under chronic stress conditions

Long-term ovarian hormone deprivation markedly increased the percent time spent immobile and decreased the percent time spent swimming in the FST, compared to sham controls. This indicates that under conditions of chronic stress, ovarian hormones may afford resilience against the development of behavioral despair or depressive-like behavior. Our findings are in partial agreement with a study indicating that long, but not short, term ovarian hormone deprivation increased immobility in the FST in mice subjected to CUS (Lagunas et al., 2010a). However, much of the previous literature on the role of ovarian hormones in depressive-like behavior is comprised of studies in which animals are not subjected to any prior stress manipulations or model of depression (Li et al., 2014; Rachman et al., 1998; Récamier-Carballo et al., 2012). While these studies demonstrate that short term ovariectomy alone (1-5 weeks) increased immobility in the FST, long-term ovariectomy (3 - 15 months) does not alter immobility in the FST (de Chaves et al., 2009; Estrada-Camarena et al., 2011). Together these past findings suggest that the increase in depressive-like behavior as a result of ovariectomy alone is transitory, and taken together with our current data, this indicates that long-term ovarian hormone deprivation results in a state of enhanced vulnerability to depressive-like endophenotypes in the face of chronic stress.

In a similar manner, in the present study we found that long-term ovarian hormone deprivation increased anxiety-like behavior in the novelty suppressed feeding test, in comparison with sham controls. This is in concert with the previously established anxiolytic effects of estradiol and estrogen receptor agonists (Lund et al., 2005; Walf and Frye, 2005), and with studies indicating enhanced anxiety-like behavior with longer periods of ovarian hormone depletion (Lagunas et al., 2010b; Picazo et al., 2006). Interestingly, we observe a trend for fluoxetine to increase latency to feed in the sham but not OVX group. This is not completely surprising given previous reports suggesting anxiogenic effects of fluoxetine treatment (Belzung et al., 2001; Leuner et al., 2004; Silva et al., 1999). Interestingly, one study found that chronic fluoxetine treatment increased anxiety-like behavior in female but not male rats (Leuner et al., 2004). This sex difference coupled with our current data suggests that ovarian hormones may be implicated in the anxiogenic effects of fluoxetine, however this requires further investigation.

In line with our other behavioral findings, sucrose preference was also significantly reduced by long-term ovariectomy. However, because there were no differences in basal sucrose preference prior to CUS, it appears that long-term ovariectomy does not itself produce

anhedonia-like behavior, but rather increases the susceptibility to anhedonia-like behavior under chronic stress. Importantly, this effect was not seen at all time points, however this may not be surprising given that sucrose anhedonia is less robust in female compared to male rodents (Grippe et al., 2005; Dalla et al., 2005, 2008; Kamper et al., 2009). Regardless of ovarian status, fluoxetine treatment resulted in a significant increase in sucrose preference after the last week of CUS, but had no significant effect on behavior in any other test (NSF and FST). This indicates that in middle-aged female rats, a 5mg/kg dose of fluoxetine may be efficacious in alleviating some but not all depressive-like behavioral phenotypes. Our findings are somewhat inconsistent with another study showing that non-efficacious doses of fluoxetine and estradiol, when administered in concert, act synergistically to reduce immobility in the FST in ovariectomized middle-aged female rats (Récamier-Carballo et al., 2012). However, important differences between the study by Recamier-Carballo et al. and ours may explain the inconsistencies in findings. These differences include the length of ovarian hormone deprivation (4 months in the current study versus 3 weeks in Recamier-Carballo et al., 2012), treatment regimen (3 weeks of fluoxetine treatment in our study versus 3 injections within 24 hours in Recamier-Carballo et al., 2012), and condition (rats were exposed to chronic stress in our study but were stress-naïve in Recamier-Carballo et al., 2012). Our data is consistent with previous studies in which estradiol is shown to increase resilience; for example, estradiol replacement in ovariectomized rats reduced the incidence of helplessness after inescapable shock relative to vehicle treatment (Bredemann and McMahon, 2014). In addition, estradiol increases cognitive resilience in the face of repeated stress (Wei et al., 2013). Collectively, findings from our study and others show that ovarian hormone deprivation may result in a state of enhanced vulnerability to the deleterious effects of chronic stress compared to sham controls.

It is important to note that it is not possible to ascertain the reason behind the lack of behavioural efficacy of fluoxetine treatment in the FST or NSF from the current data. In order to address this caveat, future work in middle-aged female rats should compare the response to additional doses of fluoxetine, and/or other SSRIs. However, it is also important to note that although SSRIs are the first-line pharmacological treatment for major depression, only a third of patients will respond to these drugs following initial treatment (Kornstein et al., 2013; Trivedi et al., 2006). Even in patients that are initially responsive to treatment, up to 57% will have depressive symptoms return due to a loss of drug efficacy. Thus, our findings are at least

somewhat consistent with the clinical literature, and perhaps it is not surprising that we see limited behavioral efficacy with SSRIs in this report.

4.2. 17 β -estradiol was associated with a facilitated antidepressant-like effect of fluoxetine to decrease immobility in the forced swim test

Higher levels of serum 17 β -estradiol were positively correlated with reduced immobility in the FST only in the fluoxetine treated sham group (OVX fluoxetine group had undetectable estradiol levels). Because the same relationship was not seen in the vehicle treated sham group, this suggests that in chronically stressed female rats, higher levels of endogenous 17 β -estradiol facilitate the effect of fluoxetine to reduce immobility in the FST. Our findings are in line with a previous report demonstrating that sub-optimal doses of 17 β -estradiol and fluoxetine administered to OVX rats act synergistically to reduce immobility in the FST (Récamier-Carballo et al., 2012). Our findings also parallel the human literature, which indicates that in post-menopausal women with depression, SSRIs are more efficacious in alleviating symptoms when prescribed in conjunction with hormone therapy (HT; Thase 2005), however other studies have failed to show this effect (Kornstein et al., 2013). The mechanisms that underlie the ovarian hormone-mediated enhancement of antidepressant efficacy are not clearly delineated. However, estradiol increases serotonergic tone via enhanced serotonin synthesis (Bethea et al., 2000), reduced degradation (Gundlah et al., 2002), decreased autoreceptor-mediated inhibition (Lu and Bethea, 2002), and serotonin transporter levels (Frokjaer et al., 2015); thus such mechanisms may be at play in the mediation of antidepressant effects.

4.3. Ovarian hormones imparted resilience against glucocorticoid-dependent negative feedback impairment

Coinciding with our behavioral results, long-term OVX rats that had undergone CUS exhibited impaired HPA negative feedback compared to the sham rats in the dexamethasone suppression test. This finding signifies that ovarian hormones may protect against HPA negative feedback impairment in the context of chronic stress. In general, ovarian hormones potentiate acute stress-induced activation of the HPA axis (Burgess and Handa, 1992) and impair HPA negative feedback inhibition (Weiser and Handa, 2009). These findings may initially seem contradictory to our current data in which the DEX suppression of CORT release is reduced in OVX rats. However, a critical consideration is that in the aforementioned studies, the subjects

were not previously exposed to chronic stress. Thus, these findings coupled with our own suggest that the role of ovarian hormones to regulate HPA axis negative feedback may be drastically affected by chronic stress. Intriguingly, our findings translate to a study of pre- and post-menopausal women, all of whom were diagnosed with depression, in which resistance to DEX suppression was found in a significantly higher proportion of postmenopausal women as compared to premenopausal women (Young et al., 1993). Furthermore our data are also consistent with the finding that estradiol improved HPA negative feedback function after DEX suppression test in women made hypogonadal (Lee et al., 2012). Lastly, we found that fluoxetine was able to improve HPA negative feedback inhibition but only in Sham and not ovariectomized controls. To our knowledge there are no studies examining antidepressant efficacy in depressed women on HPA negative feedback regulation with differing estradiol levels. Taken together, this suggests that ovarian hormones may protect against HPA axis negative feedback dysregulation in women with depression, and similarly as a result of chronic stress in female rodents.

In the present study, in OVX rats, basal levels of serum CORT were reduced, but the percent change in basal CORT did not change throughout CUS, while it was increased in Sham controls. This finding is in concert with prior research indicating that ovarian hormones exert stimulatory effects on basal HPA axis activity (Goel et al., 2014) for review).

4.4. Fluoxetine treatment increased neurogenesis and PSA-NCAM in a region specific and ovarian status-dependent manner

In the present study we found no significant differences in levels of cell proliferation (Ki67 expression), neurogenesis (BrdU/NeuN), or PSA-NCAM expression in the dentate gyrus of vehicle treated sham and long-term OVX middle-aged rats. These findings are in accordance with previous literature showing long-term ovariectomy (3-4 weeks) results in no significant change in neurogenesis (Tanapat et al., 1999) or PSA-NCAM expression (Banasr et al., 2001), unlike short-term (1 week) ovariectomy in young adult rats. Our work extends these findings to middle aged females. The return of neurogenesis levels following longer periods of ovariectomy is unlikely related to the *de novo* synthesis of estrogens in the brain, as after three weeks of ovariectomy, levels of estradiol in the hippocampus of adult female rats are not detectable (Barker and Galea, 2009), and hippocampal estradiol correlates with plasma estradiol (Kato et al., 2013). Thus, alternative mechanisms may be at play, such as ovariectomy-induced reductions

in basal corticosterone (Goel et al., 2014), which may be linked to the normalization of neurogenesis levels after long term ovariectomy. Together, our results indicate that in middle age, long-term deprivation of ovarian hormones does not alter neurogenesis or PSA-NCAM expression in the dentate gyrus.

Fluoxetine treatment increased cell proliferation, neurogenesis, and the expression of PSA-NCAM within the dentate gyrus. These findings are in line with prior reports indicating that chronic treatment with fluoxetine upregulates the same markers (Guirado et al., 2012; Malberg et al., 2000). While in older age, fluoxetine does not increase neurogenesis in the dentate gyrus (Couillard-Despres, 2009), our findings are consistent with others showing that fluoxetine upregulated PSA-NCAM in middle-aged male rats (Guirado et al., 2012). Interestingly, our current data shows that fluoxetine treatment, when initiated after BrdU administration, increased adult hippocampal neurogenesis, unlike what is seen in stress-naïve, young adult male rats (Malberg et al., 2000). Indeed, our finding is consistent with another study using young adult female rats (Vega-Rivera et al., 2015), perhaps indicating a sex difference in the response of fluoxetine administration on survival of BrdU-labelled cells. Interestingly, the fluoxetine-mediated upregulation of cell proliferation, neurogenesis, and PSA-NCAM was seen despite its lack of behavioral efficacy in the majority of tests, suggesting that these neural changes are not necessarily synonymous with behavioral efficacy, partially consistent with the existing literature in young adult male rats (Bessa et al., 2008; Santarelli, 2003). The 5mg/kg dose of fluoxetine utilized in this study has been previously shown to increase cell proliferation in adult female rats relative to a higher dose of 10mg/kg, but not compared to vehicle treatment, and the study did not examine dorsal versus ventral dentate gyrus (Pawluski et al., 2014). The same study found this differential effect of dose on cell proliferation only when fluoxetine was administered via osmotic minipump, but not orally via wafer cookie (Pawluski et al., 2014). Future studies should consider the effects fluoxetine administration route and dose on cell proliferation in different regions of the hippocampus.

Somewhat surprisingly, the effect of fluoxetine treatment to enhance cell proliferation (Ki67-ir cells) was seen in both the dorsal and ventral hippocampus of OVX rats, but only in the ventral hippocampus of sham rats. Similarly fluoxetine-mediated enhancement of neurogenesis and PSA-NCAM was exclusive to OVX rats, in the dorsal and ventral hippocampus, respectively. Thus contrary to our initial expectations, the potential for plasticity in response to

fluoxetine treatment is not reduced with long-term ovarian hormone deprivation, but rather appears to be enhanced. The mechanism for this unexpected finding is not clear but may be due to HPA-HPG interactions; because ovarian steroids have stimulatory effects on the HPA axis (Viau and Meaney, 1991) the higher levels of basal cortisol in sham rats may interact with fluoxetine to prevent the upregulation of cell proliferation that is seen in ovariectomized rats. Interestingly, fluoxetine has been shown to increase the neurosteroid allopregnanolone in the female rat brain (Fry et al., 2014), therefore it would be interesting to investigate potential modulatory effects of ovarian hormones and subsequent effects on hippocampal neurogenesis. These findings are also in partial contrast to those showing that an acute dose of estradiol with a suboptimal dose of fluoxetine can increase cell proliferation in stress-naïve female Wistar rats (Vega-Rivera et al., 2015). Considering that in the present study there were no significant differences in the effects of fluoxetine on behavior between OVX and sham groups, the functional consequences of this differential upregulation of neuroplasticity based on ovarian hormone milieu remain elusive. A region specific influence of fluoxetine in CUS exposed Sham rats on neurogenesis is perhaps not surprising given that CUS decreases neurogenesis in the ventral hippocampus and is reversed by chronic antidepressant treatment (Tanti and Belzung, 2013).

Interestingly, fluoxetine treatment improved HPA negative feedback regulation in sham rats, but had more profound pro-neurogenic effects in OVX rats. This is somewhat inconsistent with previous research in young adult male rodents, in which newly generated hippocampal neurons were shown to be required for antidepressants to improve HPA axis regulation (Surget et al., 2011), and data indicating that hippocampal neurogenesis may be important for HPA negative feedback regulation (Snyder et al., 2011). This discrepancy is interesting, as it suggests that the relationship between hippocampal neurogenesis and HPA axis function may differ between males and females. Indeed, the fact that fluoxetine only increased cell proliferation in the ventral, but not dorsal hippocampus in sham animals, unlike what occurred in OVX animals, may explain the reason for better efficacy of fluoxetine in Sham rats in the DEX suppression test. In addition, it is also possible that the fluoxetine-induced increase in neurogenesis in the dorsal hippocampus of OVX rats may have interfered with the efficacy of fluoxetine. Finally, it is important to note that it is not without precedent that neurogenesis may have different functions or links to behavior/stress in males versus females, as sex differences in neurogenesis have been

noted in response to spatial learning (Chow et al., 2013; Yagi et al., 2015), trace eyeblink conditioning (Dalla et al., 2009), acute and chronic stress (Falconer and Galea, 2003; Westenbroek et al., 2004), adolescent stress exposure (Barha et al., 2011) and chronic estradiol treatment (Barker and Galea, 2008).

4.5. Fluoxetine treatment reduced microglial number in the ventral dentate gyrus but had no effect on microglial morphology

Regardless of ovarian status, fluoxetine treatment significantly reduced the number of Iba-1-ir microglial cells in the ventral dentate gyrus, partially consistent with previous findings showing imipramine treatment reverses the initial increase and subsequent decline in microglial number as a result of chronic unpredictable stress (Kreisel et al., 2013b). However, our data provide no evidence for alterations in microglial activation with fluoxetine treatment, as there was no effect of fluoxetine on the number or length of processes in Iba-1-ir cells. Thus, our findings suggest that SSRI treatment may be associated with a reduction in microglia-mediated neuroinflammation, however this requires further investigation as microglial function depends on its activational state (Kettenmann et al., 2011) and levels of pro- versus anti-inflammatory cytokines. Importantly, our findings are in partial agreement with human literature suggesting that antidepressant treatment is associated with a reduction of peripheral inflammation, as measured by reduced pro-inflammatory cytokines such as IL-6 (Basterzi et al., 2005) and TNF- α (Tuglu et al., 2003), and confirmed by a meta-analysis (Eller et al., 2008). Taken together with our present findings, this suggests that antidepressants may exert their effects, at least in part, through the amelioration of microglia-mediated neuroinflammation in the hippocampus.

Our findings reveal no basal differences in the number or morphology of Iba-1-ir microglial cells between OVX and sham groups. This is somewhat surprising considering that ovarian hormones inhibit microglial proliferation *in vivo* (Ganter et al., 1992), and can directly alter microglial function and morphology both *in vivo* and *in vitro* (Habib and Beyer, 2015). Our findings are in contrast with a study of middle-aged female rats, in which Iba-1 mRNA expression in the hippocampus was significantly increased when measured approximately 40 days after ovariectomy (Sárvári et al., 2014). However, in our present study, the period of ovarian hormone deprivation was substantially longer (4 months), and thus compensatory mechanisms may be at play to return baseline levels of Iba-1 expression.

4.6. The physiological effects of fluoxetine were dependent on ovarian status

Fluoxetine treatment reduces body weight or attenuates weight gain in rodents (McGuirk et al., 1992). In the present study, fluoxetine reduced body weight in OVX rats only. Although the functional outcome of fluoxetine-induced weight reduction is unknown, ovarian hormones appear to protect against this effect.

5. CONCLUSIONS

In summary, ovarian hormones afforded resilience against the development of a depressive-like phenotype in middle-aged female rats exposed to chronic unpredictable stress. Long-term ovariectomy increased depressive-, and anxiety-like behaviors in the forced swim, novelty suppressed feeding, and sucrose preference tests after exposure to chronic unpredictable stress. Further, long-term ovariectomy resulted in significant impairments in the HPA negative feedback system, as demonstrated by the dexamethasone suppression test. Thus, our behavioral and endocrine findings converge to suggest that ovarian hormones are key determinants of stress resilience. With the exception of the sucrose preference test and HPA dysregulation, chronic fluoxetine treatment at 5mg/kg did not produce anti-depressant-like effects, regardless of ovarian status. However, higher levels of endogenous estradiol were associated with a facilitation of fluoxetine to decrease immobility in the forced swim test. Despite its limited efficacy, fluoxetine treatment was associated with reduced microglial expression in the ventral dentate gyrus, and increased cell proliferation, neurogenesis, and PSA-NCAM expression; effects that were varied by ovarian status and region. Our findings reinforce the need for considering the role of ovarian hormones in stress-resilience, depression, antidepressant efficacy, and the neural correlates of antidepressant use in females.

Role of the funding source

Financial support for this research was provided by an operating grant from the Canadian Institutes for Health Research (MOP142308) to LAMG. The funding source had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Acknowledgements

The authors thank Carmen Chow, Lucille Hoover, Anne Cheng, and Alice Chan for their assistance with this work.

A	Behavior			Endocrine	Neural				
	FST Immobility	NSF Latency	SPT preference	DEX suppression	Ki67	BrdU	PSA- NCAM	Iba-1 number	Iba-1 length of processes
OVX (Relative to sham)	↑	↑	↓*	↓	=	=	=	=	=

B	Behavior			Endocrine	Neural									
	FST Immobility	NSF Latency	SPT Sucrose preference	DEX suppression	Ki67		BrdU		PSA- NCAM		Iba-1 number		Iba-1 length of processes	
					D	V	D	V	D	V	D	V	D	V
Sham (FLX relative to Veh)	=	=	↑**	↓	=	↑	=	=	=	=	=	↓	=	=
OVX (FLX relative to Veh)	=	=	↑**	=	↑	↑	↑	=	=	↑	=	↓	=	=

Figure 7. Schematic summary of behavioral, endocrine, and neural findings. **(A)** The effect of long-term ovariectomy in comparison to sham controls. **(B)** The effect of fluoxetine treatment compared to vehicle treatment in each ovarian hormone condition. OVX: ovariectomy, FLX: fluoxetine, Veh: vehicle, FST: Forced Swim Test, NSF: Novelty Suppresses Feeding, SPT: Sucrose Preference Test, DEX= dexamethasone. ↑ indicates significant increase, ↓ indicates significant decrease, = indicates no significant change. * indicates significance only in week 4 of chronic unpredictable stress, ** indicates significance only in week 6 of chronic unpredictable stress.

860 **Table 1.** Stressors included in the Chronic Unpredictable Stress paradigm.

Stressor	Duration	Description
Wet bedding	2 hours	200 ml of tap water in home cage, rats moved to a dry and clean cage afterwards
Food and water deprivation	4 hours	Rats deprived of food and water, always prior to the sucrose preference test
Tail pinch	5 mins	Plastic clothespin placed at base of tail
Cage tilt	2 hours	Home cage tilted at 45°
Elevated platform	5 mins	Rats placed on a plexiglass platform (20x20 cm) at 90 cm above ground.
Continuous lighting	36 hours	Colony lights on for 36 hours
Restraint	1 hours	Rats restrained in well ventilated plexiglass tubes
Social isolation	18 hours	Rats individually housed from 15:00-10:00
Soiled cage	2 hours	Rat pairs placed in cage soiled by other rats. Rats moved to a clean cage afterwards
White noise	1 hour	Rats exposed to 80 dB of white noise
Strobing light	1 hour	Strobing light at 1flash/second in an otherwise dark room, during light cycle
Tail bleed	2 mins	Blood sample taken via tail vein nick
Food deprivation	16 hour	Rats deprived of food but not water

861 Two stressors were presented daily, in a pseudo-random order, for a duration of 6 weeks.

862

863 **Table 2.** Mean ($\pm$ SEM) percentage of BrdU/NeuN colabeled cells in the granule cell layer of the
864 dentate gyrus of all groups.

Ovarian status	Drug treatment	% of BrdU/NeuN colabeled cells
Sham	Veh	77.50 ± 6.77
	FLX	75.85 ± 4.90
OVX	Veh	75.35 ± 7.08
	FLX	77.54 ± 4.65

865 The groups did not significantly differ on the average percentage of BrdU/NeuN colabeled cells
866 (all p 's>0.75). Veh= vehicle, FLX=fluoxetine, OVX=ovariectomy.

867 **Table 3.** Mean ($\pm$ SEM) length of processes of Iba-1 immunoreactive cells in the granule cell
868 layer of the dorsal and ventral dentate gyrus of all groups.

Ovarian status	Drug treatment	Average length of processes	
		Dorsal	Ventral
Sham	Veh	149.22 ± 4.76	158.73 ± 4.33
	FLX	152.06 ± 5.05	157.63 ± 4.91
OVX	Veh	154.15 ± 4.11	162.01 ± 6.18
	FLX	159.40 ± 2.65	162.59 ± 4.01

869 The average length of processes was significantly higher in the ventral dentate gyrus relative to
870 the dorsal dentate gyrus ($p=0.014$), but there were no other significant main effects or
871 interactions (all p 's>0.19). Veh= vehicle, FLX=fluoxetine, OVX=ovariectomy.

References

- Ahokas, A., Kaukoranta, J., Wahlbeck, K., Aito, M., 2001. Estrogen deficiency in severe postpartum depression: successful treatment with sublingual physiologic 17beta-estradiol: a preliminary study. *J Clin Psychiatry* 62, 332–336.
- Banasr, M., Hery, M., Brezun, J.M., Daszuta, A., 2001. Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. *Eur. J. Neurosci.* 14, 1417–1424.
- Banasr, M., Hery, M., Printemps, R., Daszuta, A., 2004. Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacology* 29, 450–460. doi:10.1038/sj.npp.1300320
- Barha, C.K., Brummelte, S., Lieblich, S.E., Galea, L.A.M., 2011. Chronic restraint stress in adolescence differentially influences hypothalamic-pituitary-adrenal axis function and adult hippocampal neurogenesis in male and female rats. *Hippocampus* 21, 1216–1227. doi:10.1002/hipo.20829
- Barha, C.K., Dalton, G.L., Galea, L.A., 2010. Low doses of 17alpha-estradiol and 17beta-estradiol facilitate, whereas higher doses of estrone and 17alpha- and 17beta-estradiol impair, contextual fear conditioning in adult female rats. *Neuropsychopharmacology* 35, 547–559. doi:10.1038/npp.2009.161
- Barha, C.K., Galea, L.A.M., 2013. The hormone therapy, Premarin, impairs hippocampus-dependent spatial learning and memory and reduces activation of new granule neurons in response to memory in female rats. *Neurobiology of Aging* 34, 986–1004. doi:10.1016/j.neurobiolaging.2012.07.009
- Barker, J.M., Galea, L.A.M., 2009. Sex and regional differences in estradiol content in the prefrontal cortex, amygdala and hippocampus of adult male and female rats. *Gen. Comp. Endocrinol.* 164, 77–84. doi:10.1016/j.ygcen.2009.05.008
- Barker, J.M., Galea, L.A.M., 2008. Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats. *Neuroscience* 152, 888–902. doi:10.1016/j.neuroscience.2007.10.071
- Basterzi, A.D., Aydemir, C., Kisa, C., Aksaray, S., Tuzer, V., Yazici, K., Göka, E., 2005. IL-6 levels decrease with SSRI treatment in patients with major depression. *Hum Psychopharmacol* 20, 473–476. doi:10.1002/hup.717
- Bekku, N., Yoshimura, H., 2005. Animal model of menopausal depressive-like state in female mice: prolongation of immobility time in the forced swimming test following ovariectomy. *Psychopharmacology (Berl.)* 183, 300–307. doi:10.1007/s00213-005-0179-0
- Belzung, C., Le Guisquet, A.M., Barreau, S., Calatayud, F., 2001. An investigation of the mechanisms responsible for acute fluoxetine-induced anxiogenic-like effects in mice. *Behav Pharmacol* 12, 151–162.
- Bessa, J.M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J.J., Palha, J.A., Almeida, O.F.X., Sousa, N., 2008. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling 14, 764–773. doi:10.1038/mp.2008.119
- Bethea, C.L., Mirkes, S.J., Shively, C.A., Adams, M.R., 2000. Steroid regulation of tryptophan hydroxylase protein in the dorsal raphe of macaques. *BPS* 47, 562–576.
- Binder, E.B., Künzel, H.E., Nickel, T., Kern, N., Pfennig, A., Majer, M., Uhr, M., Ising, M., Holsboer, F., 2009. HPA-axis regulation at in-patient admission is associated with

antidepressant therapy outcome in male but not in female depressed patients. *Psychoneuroendocrinology* 34, 99–109. doi:10.1016/j.psyneuen.2008.08.018

Boldrini, M., Hen, R., Underwood, M.D., Rosoklija, G.B., Dwork, A.J., Mann, J.J., Arango, V., 2012. Hippocampal Angiogenesis and Progenitor Cell Proliferation Are Increased with Antidepressant Use in Major Depression. *Biological Psychiatry* 72, 562–571. doi:10.1016/j.biopsych.2012.04.024

Bredemann, T.M., McMahon, L.L., 2014. 17 β Estradiol increases resilience and improves hippocampal synaptic function in helpless ovariectomized rats. *Psychoneuroendocrinology* 42, 77–88. doi:10.1016/j.psyneuen.2014.01.004

Brummelte, S., Galea, L.A.M., 2010. Chronic high corticosterone reduces neurogenesis in the dentate gyrus of adult male and female rats. *Neuroscience* 168, 680–690. doi:10.1016/j.neuroscience.2010.04.023

Burgess, L.H., Handa, R.J., 1992. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* 131, 1261–1269. doi:10.1210/endo.131.3.1324155

Cameron, H.A., Woolley, C.S., McEwen, B.S., Gould, E., 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56, 337–344.

Chow, C., Epp, J.R., Lieblisch, S.E., Barha, C.K., Galea, L.A.M., 2013. Sex differences in neurogenesis and activation of new neurons in response to spatial learning and memory. *Psychoneuroendocrinology* 38, 1236–1250. doi:10.1016/j.psyneuen.2012.11.007

Cohen, L.S., Soares, C.N., Vitonis, A.F., Otto, M.W., Harlow, B.L., 2006. Risk for New Onset of Depression During the Menopausal Transition: The Harvard Study of Moods and Cycles. *Arch Gen Psychiatry* 63, 385–390. doi:10.1001/archpsyc.63.4.385

Dalla, C., Papachristos, E.B., Whetstone, A.S., Shors, T.J., 2009. Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2927–2932. doi:10.1073/pnas.0809650106

de Chaves, G., Moretti, M., Castro, A.A., Dagostin, W., da Silva, G.G., Boeck, C.R., Quevedo, J., Gavioli, E.C., 2009. Effects of long-term ovariectomy on anxiety and behavioral despair in rats. *Physiology & Behavior* 97, 420–425. doi:10.1016/j.physbeh.2009.03.016

Eller, T., Vasar, V., Shlik, J., Maron, E., 2008. Pro-inflammatory cytokines and treatment response to escitalopram in major depressive disorder. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 445–450. doi:10.1016/j.pnpbp.2007.09.015

Epp, J.R., Beasley, C.L., Galea, L.A., 2013. Increased Hippocampal Neurogenesis and p21 Expression in Depression: Dependent on Antidepressants, Sex, Age, and Antipsychotic Exposure. *Neuropsychopharmacology* 38, 2297–2306. doi:10.1038/npp.2013.132

Estrada-Camarena, E., López-Rubalcava, C., Hernández-Aragón, A., Mejía-Mauries, S., Picazo, O., 2011. Long-term ovariectomy modulates the antidepressant-like action of estrogens, but not of antidepressants. *J. Psychopharmacol. (Oxford)* 25, 1365–1377. doi:10.1177/0269881111408456

Falconer, E.M., Galea, L.A.M., 2003. Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Research* 975, 22–36.

Fanselow, M.S., Dong, H.-W., 2010. Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron* 65, 7–19. doi:10.1016/j.neuron.2009.11.031

Frank, M.G., Baratta, M.V., Sprunger, D.B., Watkins, L.R., Maier, S.F., 2007. Microglia serve as

964 a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine
 965 responses. *Brain Behavior and Immunity* 21, 47–59. doi:10.1016/j.bbi.2006.03.005
 966 Frokjaer, V.G., Pinborg, A., Holst, K.K., Overgaard, A., Henningsson, S., Heede, M., Larsen,
 967 E.C., Jensen, P.S., Agn, M., Nielsen, A.P., Stenbæk, D.S., da Cunha-Bang, S., Lehel, S.,
 968 Siebner, H.R., Mikkelsen, J.D., Svarer, C., Knudsen, G.M., 2015. Role of Serotonin
 969 Transporter Changes in Depressive Responses to Sex-Steroid Hormone Manipulation: A
 970 Positron Emission Tomography Study. *Biological Psychiatry*.
 971 doi:10.1016/j.biopsych.2015.04.015
 972 Fry, J.P., Li, K.Y., Devall, A.J., Cockcroft, S., Honour, J.W., Lovick, T.A., 2014. Fluoxetine
 973 elevates allopregnanolone in female rat brain but inhibits a steroid microsomal
 974 dehydrogenase rather than activating an aldo-keto reductase. *Br. J. Pharmacol.* 171, 5870–
 975 5880. doi:10.1111/bph.12891
 976 Galea, L.A., Wide, J.K., Barr, A.M., 2001. Estradiol alleviates depressive-like symptoms in a
 977 novel animal model of post-partum depression. *Behavioural Brain Research* 122, 1–9.
 978 Ganter, S., Northoff, H., Männel, D., Gebicke-Härter, P.J., 1992. Growth control of cultured
 979 microglia. *J. Neurosci. Res.* 33, 218–230. doi:10.1002/jnr.490330205
 980 Goel, N., Workman, J.L., Lee, T.T., Innala, L., Viau, V., 2014. Sex differences in the HPA axis.
 981 *Compr Physiol* 4, 1121–1155. doi:10.1002/cphy.c130054
 982 Green, A.D., Barr, A.M., Galea, L.A.M., 2009. Role of estradiol withdrawal in “anhedonic”
 983 sucrose consumption: a model of postpartum depression. *Physiology & Behavior* 97, 259–
 984 265. doi:10.1016/j.physbeh.2009.02.020
 985 Green, A.D., Galea, L.A.M., 2008. Adult hippocampal cell proliferation is suppressed with
 986 estrogen withdrawal after a hormone-simulated pregnancy. *Hormones and Behavior* 54,
 987 203–211. doi:10.1016/j.yhbeh.2008.02.023
 988 Guirado, R., Sanchez-Matarredona, D., Varea, E., Crespo, C., Blasco-Ibáñez, J.M., Nacher, J.,
 989 2012. Chronic fluoxetine treatment in middle-aged rats induces changes in the expression of
 990 plasticity-related molecules and in neurogenesis. *BMC Neurosci* 13, 5. doi:10.1186/1471-
 991 2202-13-5
 992 Gundlah, C., Lu, N.Z., Bethea, C.L., 2002. Ovarian steroid regulation of monoamine oxidase-A
 993 and -B mRNAs in the macaque dorsal raphe and hypothalamic nuclei. *Psychopharmacology*
 994 (Berl.) 160, 271–282. doi:10.1007/s00213-001-0959-0
 995 Gutiérrez-Lobos, K., Scherer, M., Anderer, P., Katschnig, H., 2002. The influence of age on the
 996 female/male ratio of treated incidence rates in depression. *BMC Psychiatry* 2, 3.
 997 doi:10.1186/1471-244X-2-3
 998 Habib, P., Beyer, C., 2015. Regulation of brain microglia by female gonadal steroids. *Journal of*
 999 *Steroid Biochemistry and Molecular Biology* 146, 3–14. doi:10.1016/j.jsbmb.2014.02.018
 1000 Hamson, D.K., Wainwright, S.R., Taylor, J.R., Jones, B.A., Watson, N.V., Galea, L.A.M., 2013.
 1001 Androgens increase survival of adult-born neurons in the dentate gyrus by an androgen
 1002 receptor-dependent mechanism in male rats. *Endocrinology* 154, 3294–3304.
 1003 doi:10.1210/en.2013-1129
 1004 Hendrick, V., Altshuler, L.L., Suri, R., 1998. Hormonal Changes in the Postpartum and
 1005 Implications for Postpartum Depression. *Psychosomatics* 39, 93–101. doi:10.1016/S0033-
 1006 3182(98)71355-6
 1007 Ising, M., Horstmann, S., Kloiber, S., Lucae, S., Binder, E.B., Kern, N., Kunzel, H.E., Pfennig,
 1008 A., Uhr, M., Holsboer, F., 2007. Combined Dexamethasone/Corticotropin Releasing
 1009 Hormone Test Predicts Treatment Response in Major Depression—A Potential Biomarker?

- Biological Psychiatry 62, 47–54. doi:10.1016/j.biopsych.2006.07.039
- Kato, A., Hojo, Y., Higo, S., Komatsuzaki, Y., Murakami, G., Yoshino, H., Uebayashi, M., Kawato, S., 2013. Female hippocampal estrogens have a significant correlation with cyclic fluctuation of hippocampal spines. *Front Neural Circuits* 7, 149. doi:10.3389/fncir.2013.00149
- Kettenmann, H., Hanisch, U.-K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. *Physiological Reviews* 91, 461–553. doi:10.1152/physrev.00011.2010
- Kondratiuk, I., Plucinska, G., Mischczuk, D., Wozniak, G., Szydlowska, K., Kaczmarek, L., Filipkowski, R.K., Lukasiuk, K., 2015. Epileptogenesis following Kainic Acid-Induced Status Epilepticus in Cyclin D2 Knock-Out Mice with Diminished Adult Neurogenesis. *PLoS ONE* 10, e0128285. doi:10.1371/journal.pone.0128285
- Kornstein, S.G., Toups, M., Rush, A.J., Wisniewski, S.R., Thase, M.E., Luther, J., Warden, D., Fava, M., Trivedi, M.H., 2013. Do menopausal status and use of hormone therapy affect antidepressant treatment response? Findings from the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study. *J Womens Health (Larchmt)* 22, 121–131. doi:10.1089/jwh.2012.3479
- Kreisel, T., Frank, M.G., Licht, T., Reshef, R., Ben-Menachem-Zidon, O., Baratta, M.V., Maier, S.F., Yirmiya, R., 2013a. Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Molecular Psychiatry* 19, 699–709. doi:10.1038/mp.2013.155
- Kreisel, T., Frank, M.G., Licht, T., Reshef, R., Ben-Menachem-Zidon, O., Baratta, M.V., Maier, S.F., Yirmiya, R., 2013b. Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Molecular Psychiatry* 19, 699–709. doi:10.1038/mp.2013.155
- Kuhn, H.G., Dickinson-Anson, H., Gage, F.H., 1996. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *Journal of Neuroscience* 16, 2027–2033.
- Lagunas, N., Calmarza-Font, I., Diz-Chaves, Y., Garcia-Segura, L.M., 2010a. Long-term ovariectomy enhances anxiety and depressive-like behaviors in mice submitted to chronic unpredictable stress. *Hormones and Behavior* 58, 786–791. doi:10.1016/j.yhbeh.2010.07.014
- Lagunas, N., Calmarza-Font, I., Diz-Chaves, Y., Garcia-Segura, L.M., 2010b. Long-term ovariectomy enhances anxiety and depressive-like behaviors in mice submitted to chronic unpredictable stress. *Hormones and Behavior* 58, 786–791. doi:10.1016/j.yhbeh.2010.07.014
- Lee, E.E., Nieman, L.K., Martinez, P.E., Harsh, V.L., Rubinow, D.R., Schmidt, P.J., 2012. ACTH and cortisol response to Dex/CRH testing in women with and without premenstrual dysphoria during GnRH agonist-induced hypogonadism and ovarian steroid replacement. *The Journal of Clinical Endocrinology & Metabolism* 97, 1887–1896. doi:10.1210/jc.2011-3451
- Leuner, B., Mendolia-Loffredo, S., Shors, T.J., 2004. Males and females respond differently to controllability and antidepressant treatment. *BPS* 56, 964–970. doi:10.1016/j.biopsych.2004.09.018
- Li, L.-H., Wang, Z.-C., Yu, J., Zhang, Y.-Q., 2014. Ovariectomy results in variable changes in nociception, mood and depression in adult female rats. *PLoS ONE* 9, e94312. doi:10.1371/journal.pone.0094312
- Lu, N.Z., Bethea, C.L., 2002. Ovarian steroid regulation of 5-HT_{1A} receptor binding and G protein activation in female monkeys. *Neuropsychopharmacology* 27, 12–24.

- doi:10.1016/S0893-133X(01)00423-7
- Lund, T.D., Rovis, T., Chung, W.C.J., Handa, R.J., 2005. Novel Actions of Estrogen Receptor- β on Anxiety-Related Behaviors. *Endocrinology* 146, 797–807. doi:10.1210/en.2004-1158
- Maes, M., Claes, M., Schotte, C., Delbeke, L., Jacquemyn, Y., Verkerk, R., De Meester, I., Scharpé, S., 1992. Disturbances in dexamethasone suppression test and lower availability of L-tryptophan and tyrosine in early puerperium and in women under contraceptive therapy. *J Psychosom Res* 36, 191–197.
- Malberg, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S., 2000. Chronic Antidepressant Treatment Increases Neurogenesis in Adult Rat Hippocampus. *Journal of Neuroscience* 20, 9104–9110. doi:10.1016/0306-4522(93)90335-D
- McGuirk, J., Muscat, R., Willner, P., 1992. Effects of chronically administered fluoxetine and fenfluramine on food intake, body weight and the behavioural satiety sequence. *Psychopharmacology (Berl.)* 106, 401–407.
- McKinnon, M.C., Yucel, K., Nazarov, A., MacQueen, G.M., 2009. A meta-analysis examining clinical predictors of hippocampal volume in patients with major depressive disorder. *J Psychiatry Neurosci* 34, 41–54.
- Miller, A.H., Maletic, V., Raison, C.L., 2009. Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. *BPS* 65, 732–741. doi:10.1016/j.biopsych.2008.11.029
- Moses-Kolko, E.L., Berga, S.L., Kalro, B., Sit, D.K.Y., Wisner, K.L., 2009. Transdermal estradiol for postpartum depression: a promising treatment option. *Clin Obstet Gynecol* 52, 516–529. doi:10.1097/GRF.0b013e3181b5a395
- Nemeth, C.L., Reddy, R., Bekhbat, M., Bailey, J., Neigh, G.N., 2014. Microglial activation occurs in the absence of anxiety-like behavior following microembolic stroke in female, but not male, rats. *J Neuroinflammation* 11, 174. doi:10.1186/s12974-014-0174-7
- Okada, M., Hayashi, N., Kometani, M., Nakao, K., Inukai, T., 1997. Influences of ovariectomy and continuous replacement of 17 β -estradiol on the tail skin temperature and behavior in the forced swimming test in rats. *Jpn. J. Pharmacol.* 73, 93–96.
- Pawluski, J.L., van Donkelaar, E., Abrams, Z., Houbart, V., Fillet, M., Steinbusch, H.W.M., Charlier, T.D., 2014. Fluoxetine dose and administration method differentially affect hippocampal plasticity in adult female rats. *Neural Plasticity* 2014, 123026–9. doi:10.1155/2014/123026
- Picazo, O., Estrada-Camarena, E., Hernández-Aragón, A., 2006. Influence of the post-ovariectomy time frame on the experimental anxiety and the behavioural actions of some anxiolytic agents. *Eur. J. Pharmacol.* 530, 88–94. doi:10.1016/j.ejphar.2005.11.024
- Rachman, I.M., Unnerstall, J.R., Pfaff, D.W., Cohen, R.S., 1998. Estrogen alters behavior and forebrain c-fos expression in ovariectomized rats subjected to the forced swim test. *PNAS* 95, 13941–13946.
- Rao, M.S., Hattiangady, B., Abdel-Rahman, A., Stanley, D.P., Shetty, A.K., 2005. Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation. *Eur. J. Neurosci.* 21, 464–476. doi:10.1111/j.1460-9568.2005.03853.x
- Rasgon, N.L., Dunkin, J., Fairbanks, L., Altshuler, L.L., Troung, C., Elman, S., Wroolie, T.E., Brunhuber, M.V., Rapkin, A., 2007. Estrogen and response to sertraline in postmenopausal women with major depressive disorder: a pilot study. *J Psychiatr Res* 41, 338–343. doi:10.1016/j.jpsychires.2006.03.009

- Récamier-Carballo, S., Estrada-Camarena, E., Reyes, R., Fernández-Guasti, A., 2012. Synergistic effect of estradiol and fluoxetine in young adult and middle-aged female rats in two models of experimental depression. *Behavioural Brain Research* 233, 351–358. doi:10.1016/j.bbr.2012.05.034
- Santarelli, L., 2003. Requirement of Hippocampal Neurogenesis for the Behavioral Effects of Antidepressants. *Science* 301, 805–809. doi:10.1126/science.1083328
- Sárvári, M., Kalló, I., Hrabovszky, E., Solymosi, N., Liposits, Z., 2014. Ovariectomy and subsequent treatment with estrogen receptor agonists tune the innate immune system of the hippocampus in middle-aged female rats. *PLoS ONE* 9, e88540. doi:10.1371/journal.pone.0088540
- Sell, S.L., Craft, R.M., Seitz, P.K., Stutz, S.J., Cunningham, K.A., Thomas, M.L., 2008. Estradiol-sertraline synergy in ovariectomized rats. *Psychoneuroendocrinology* 33, 1051–1060. doi:10.1016/j.psyneuen.2008.05.006
- Setiawan, E., Wilson, A.A., Mizrahi, R., Rusjan, P.M., Miler, L., Rajkowska, G., Suridjan, I., Kennedy, J.L., Rekkas, P.V., Houle, S., Meyer, J.H., 2015. Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry*. doi:10.1001/jamapsychiatry.2014.2427
- Silva, M.T., Alves, C.R., Santarem, E.M., 1999. Anxiogenic-like effect of acute and chronic fluoxetine on rats tested on the elevated plus-maze. *Braz. J. Med. Biol. Res.* 32, 333–339.
- Snyder, J.S., Soumier, A., Brewer, M., Pickel, J., Cameron, H.A., 2011. Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* 476, 458–460. doi:10.1038/nature10287
- Soares, C.N., 2014. Mood disorders in midlife women: understanding the critical window and its clinical implications. *Menopause* 21, 198–206. doi:10.1097/GME.0000000000000193
- Stetler, C., Miller, G.E., 2011. Depression and hypothalamic-pituitary-adrenal activation: a quantitative summary of four decades of research. *Psychosom Med* 73, 114–126. doi:10.1097/PSY.0b013e31820ad12b
- Surget, A., Tanti, A., Leonardo, E.D., Laugeray, A., Rainer, Q., Touma, C., Palme, R., Griebel, G., Ibarguen-Vargas, Y., Hen, R., Belzung, C., 2011. Antidepressants recruit new neurons to improve stress response regulation. *Molecular Psychiatry* 16, 1177–1188. doi:10.1038/mp.2011.48
- Tanapat, P., Hastings, N.B., Reeves, A.J., Gould, E., 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci.* 19, 5792–5801.
- Tanti, A., Belzung, C., 2013. Neurogenesis along the septo-temporal axis of the hippocampus: are depression and the action of antidepressants region-specific? *Neuroscience* 252, 234–252. doi:10.1016/j.neuroscience.2013.08.017
- Thase, M.E., Entsuah, R., Cantillon, M., Kornstein, S.G., 2005. Relative antidepressant efficacy of venlafaxine and SSRIs: sex-age interactions. *J Womens Health (Larchmt)* 14, 609–616. doi:10.1089/jwh.2005.14.609
- Trivedi, M.H., Rush, A.J., Wisniewski, S.R., Nierenberg, A.A., Warden, D., Ritz, L., Norquist, G., Howland, R.H., Lebowitz, B., McGrath, P.J., Shores-Wilson, K., Biggs, M.M., Balasubramani, G.K., Fava, M., STAR*D Study Team, 2006. Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. *Am J Psychiatry* 163, 28–40. doi:10.1176/appi.ajp.163.1.28
- Tuglu, C., Kara, S.H., Caliyurt, O., Vardar, E., Abay, E., 2003. Increased serum tumor necrosis

- factor-alpha levels and treatment response in major depressive disorder.
Psychopharmacology (Berl.) 170, 429–433. doi:10.1007/s00213-003-1566-z
- Vakili, K., Pillay, S.S., Lafer, B., Fava, M., Renshaw, P.F., Bonello-Cintron, C.M., Yurgelun-Todd, D.A., 2000. Hippocampal volume in primary unipolar major depression: a magnetic resonance imaging study. *BPS* 47, 1087–1090.
- Vega-Rivera, N.M., Fernández-Guasti, A., Ramírez-Rodríguez, G., Estrada-Camarena, E., 2015. Effect of sub-optimal doses of fluoxetine plus estradiol on antidepressant-like behavior and hippocampal neurogenesis in ovariectomized rats. *Psychoneuroendocrinology* 57, 113–124. doi:10.1016/j.psyneuen.2015.03.022
- Viau, V., Meaney, M.J., 1991. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* 129, 2503–2511. doi:10.1210/endo-129-5-2503
- Wainwright, S.R., Lieblich, S.E., Galea, L.A.M., 2011. Hypogonadism predisposes males to the development of behavioural and neuroplastic depressive phenotypes. *Psychoneuroendocrinology* 36, 1327–1341. doi:10.1016/j.psyneuen.2011.03.004
- Walf, A.A., Frye, C.A., 2005. ER[β]-Selective Estrogen Receptor Modulators Produce Antianxiety Behavior when Administered Systemically to Ovariectomized Rats. *Neuropsychopharmacology* 30, 1598–1609. doi:10.1038/sj.npp.1300713
- Walf, A.A., Rhodes, M.E., Frye, C.A., 2004. Antidepressant effects of ER β -selective estrogen receptor modulators in the forced swim test. *Pharmacology Biochemistry and Behavior* 78, 523–529. doi:10.1016/j.pbb.2004.03.023
- Wei, J., Yuen, E.Y., Liu, W., Li, X., Zhong, P., Karatsoreos, I.N., McEwen, B.S., Yan, Z., 2013. Estrogen protects against the detrimental effects of repeated stress on glutamatergic transmission and cognition 19, 588–598. doi:10.1038/mp.2013.83
- Weiser, M.J., Handa, R.J., 2009. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience* 159, 883–895. doi:10.1016/j.neuroscience.2008.12.058
- Westenbroek, C., Boer, Den, J.A., Veenhuis, M., Horst, Ter, G.J., 2004. Chronic stress and social housing differentially affect neurogenesis in male and female rats. *Brain Research Bulletin* 64, 303–308.
- Yagi, S., Chow, C., Lieblich, S.E., Galea, L.A.M., 2015. Sex and strategy use matters for pattern separation, adult neurogenesis, and immediate early gene expression in the hippocampus. *Hippocampus* n/a–n/a. doi:10.1002/hipo.22493
- Young, E.A., Kotun, J., Haskett, R.F., Grunhaus, L., Greden, J.F., Watson, S.J., Akil, H., 1993. Dissociation between pituitary and adrenal suppression to dexamethasone in depression. *Arch Gen Psychiatry* 50, 395–403.