OVARIAN HORMONES MODULATE ANTIDEPRESSANT-INDUCED HIPPOCAMPAL CELL PROLIFERATION IN THE DENTATE GYRUS OF ADULT FEMALE RATS

by

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ABSTRACT

Major depression is twice as common in women and symptoms are more severe in women than in men. These sex differences are linked to ovarian hormone levels in women. Interestingly, ovarian hormones may modulate antidepressant efficacy, with low ovarian hormone levels in women associated with a poorer antidepressant response. The mechanisms behind antidepressant efficacy have not yet been established, but chronic, and not acute, antidepressant treatment increases hippocampal neurogenesis in rodents and humans and normalizes hypothalamic-pituitary-adrenal axis negative feedback in rodents and humans. The antidepressant-induced increase in neurogenesis is, therefore, one mechanism by which antidepressants may work to alleviate some depressive symptoms. In this experiment we examined the effect of ovarian hormone status on the ability of chronic antidepressant treatment to increase cell proliferation in the dentate gyrus of female rats. Adult female rats were ovariectomized (OVX) or sham ovariectomized (Sham) prior to receiving 21 daily injections with either vehicle, the tricyclic antidepressant imipramine, or the selective serotonin reuptake inhibitor fluoxetine. Animals were then perfused, and brains were immunohistochemically processed for two endogenous markers: Ki67, which labels proliferating cells in the previous 24 hours, and doublecortin, which labels immature neurons aged 1-21 days. Ki67- and doublecortin-labeled cells were counted in two regions of the dentate gyrus: the dorsal region, important for memory, and the ventral region, important for regulating stress/emotion. Chronic imipramine treatment increased cell proliferation (Ki67-labeled cells) in the ventral dentate gyrus of Sham animals only, while chronic fluoxetine treatment increased cell proliferation in the dorsal dentate gyrus of OVX animals only. Furthermore, OVX animals receiving imipramine had an increased number of

immature neurons (doublecortin-labeled cells) in the ventral dentate gyrus. OVX/water compared to Sham/water controls had significantly decreased adrenal to body weight ratios that were restored following chronic treatment with imipramine and fluoxetine. Both antidepressants also lengthened the estrous cycle. This study is the first to demonstrate that ovarian hormones modulate antidepressant-induced increases in cell proliferation in females in a drug-specific and region-specific manner, and highlights the importance of considering ovarian hormone status when examining the neurogenic effects of antidepressants.

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ABBREVIATIONS

ANOVA	analysis of variance
DCX	doublecortin
DEX-CRH	dexamethasone/corticotropin-releasing hormone
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FLX	fluoxetine
G_0	resting phase of the cell cycle
G_1	gap 1 phase of the cell cycle
GCL	granule cell layer
HPA	hypothalamic-pituitary-adrenal
IMI	imipramine
OVX	ovariectomized
PBS	phosphate buffered saline
s.c.	subcutaneous
SEM	standard error of the mean
Sham	sham ovariectomized
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant

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INTRODUCTION

Major Depressive Disorder is a serious neuropsychiatric illness with a lifetime prevalence of almost 20% worldwide (Bromet et al., 2011; Kessler et al., 2012). For a diagnosis to be made, symptoms of depression, including either depressed mood or anhedonia, must persist for a minimum of two weeks and must lead to significant impairment in daily functioning (American Psychological Association, DSM-IV-TR, 2000). At least five of the following symptoms must be present: depressed mood, anhedonia, weight loss or weight gain, insomnia or hypersomnia, psychomotor agitation or retardation, loss of energy, feelings of worthlessness or guilt, decreased thinking or concentration, and suicidal thoughts. Response rates to antidepressant treatment are generally poor, with approximately 35-50% of depressed individuals experiencing treatment-resistant depression at any given time (Nemeroff, 2007). To improve the treatment of depression we must first understand the factors affecting development of depression and successful antidepressant response.

Sex differences in depression

There are pronounced sex differences in the risk to develop depression across the lifespan, with minimal sex differences prior to puberty (Angold, 2000). However, after puberty women are at least two times more likely to develop depression than men (Gutierrez-Lobos et al., 2002; Bromet et al., 2011). During adulthood, the incidence of depression in women is three times higher than in men, especially during the postpartum and perimenopausal periods (Steiner et al., 2003). The postpartum and perimenopausal periods are characterized by significant

fluctuations in ovarian hormones levels (estrogens and progesterone), suggesting that fluctuating ovarian hormones may play a role in modulating depressive vulnerability in women.

Two of the most common classes of antidepressants are selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs). SSRIs function to increase the amount of serotonin available in the synapse by inhibiting its reuptake into the presynaptic neuron, while TCAs are an older, broader-acting class of drugs that inhibit the reuptake of serotonin and norepinephrine, among other neurotransmitters (Berton and Nestler, 2006). Research suggests that there may be sex differences in antidepressant efficacy, with women responding better to SSRIs and men responding better to TCAs (Kornstein et al., 2000; Baca et al., 2004). However, this potential sex difference in antidepressant efficacy remains highly controversial, with some studies showing no sex difference in antidepressant efficacy (Parker et al., 2003; Quitkin et al., 2002; Hildebrandt et al., 2003). These discrepant findings could be due to a number of factors that are often not accounted for in individual studies; for example, there are established sex differences in severity of depression (Marcus et al., 2005), symptoms of depression (Marcus et al., 2005), side effects of antidepressants (Kornstein et al., 2000), and pharmacokinetics of antidepressants (Keers and Aitchison, 2010). Furthermore, sex differences can be modified by gonadal hormone levels (Sloan and Kornstein, 2003), but gonadal hormone levels are not usually accounted for in the aforementioned studies. Thus, individuals with differing background levels of ovarian hormones may respond differently to antidepressant treatment.

The role of ovarian hormones in depression

During the reproductive years ovarian hormone levels often fluctuate dramatically (during the menstrual cycle, pregnancy, and perimenopause), or decline substantially (during the

postpartum and menopause). Most of these life events are characterized by increased incidence and/or severity of depression, with the postpartum and perimenopausal periods considered the times of greatest risk for the development of depression during a woman's lifetime (Halbreich and Kahn, 2001; Steiner, 2003). Interestingly, women suffering from postpartum or perimenopausal depression, when ovarian hormone levels are low or declining, show greater behavioural symptom alleviation following hormone replacement therapy compared to placebo, where behavioural symptom alleviation refers to declines in the number and severity of depressive symptoms (Gregoire et al., 1996; Soares et al., 2001). Furthermore, surgically menopausal women also show a greater decline in depressive symptoms following hormone replacement therapy compared to placebo (Sherwin, 1988). Thus, supplemental ovarian hormones under low ovarian hormone conditions may help regulate depressed mood.

Ovarian hormone levels may also affect the behavioural response to antidepressants (Pae et al., 2009; Pinto-Meza et al., 2006; Grigoriadis et al., 2003; Zanardi et al., 2007). Premenopausal compared to postmenopausal women receiving antidepressants show a greater decline in the number and severity of depressive symptoms after treatment (Pae et al., 2009; Pinto-Meza et al., 2006; Grigoriadis et al., 2003). Postmenopausal women receiving hormone replacement therapy in conjunction with antidepressants also show a greater decline in depressive symptoms compared to postmenopausal women treated with antidepressants only (Zanardi et al., 2007). These findings suggest that ovarian hormone levels modulate antidepressant response, and that normal ovarian functioning may facilitate the behavioural response to antidepressants. It is important to note that the majority of this research was conducted using SSRI antidepressants. The efficacy of TCA and SSRI antidepressants may

differ in pre- and post-menopausal women, with TCAs potentially being less effective than SSRIs in pre- compared to postmenopausal women (Kornstein et al., 2000).

Ovarian and adrenal hormone interactions in depression

Stress precedes depression in approximately 80% of cases (Hammen, 2003). Furthermore, individuals with depression often show dysregulated hypothalamic-pituitary-adrenal (HPA) axis function in response to the dexamethasone/corticotropin-releasing hormone (DEX-CRH) test. Most depressed individuals exposed to the DEX-CRH test present with impaired HPA axis negative feedback that is normalized following successful treatment with TCA or SSRI antidepressants (Nickel et al., 2003; Ising et al., 2007). In fact, research shows that the ability of antidepressants to normalize HPA axis negative feedback often slightly precedes depressive symptom alleviation (Ising et al., 2007). Furthermore, there are sex differences in the ability of antidepressants to normalize HPA axis function, with remission of depressive symptoms associated with a better HPA axis response to the DEX-CRH test in women than in men (Binder et al., 2009). Interestingly, postmenopausal women show the same pattern of impaired HPA axis negative feedback seen in depressed individuals (Wolf and Kudielka, 2008), and estradiol replacement therapy can attenuate this dysregulation (Kudielka et al., 1999). Postmenopausal women also present with flattened but high cortisol levels (Wolf and Kudielka, 2008) similar to the flattened, high levels seen in depression (Stetler and Miller, 2011). These data suggest an association between ovarian hormones, HPA axis function, age, and depression, and further research examining the interplay between steroid hormones and antidepressant efficacy is warranted.

Depression and adult hippocampal neurogenesis

While research indicates that steroid hormones are involved in depression, the neurobiology of depression is not yet well understood. The monoamine theory of depression is the main theory of depression and suggests that depression is a neurochemical disorder caused by a deficit in monoamine neurotransmission. However, even though antidepressants upregulate monoamine levels within hours after exposure, alleviation of depressive symptoms takes several weeks (Berton and Nestler, 2006). Thus, alternative theories of depression must be examined, and research suggests that there are also genetic (Caspi et al., 2003), immunological (Anisman and Merali, 2002), neuroendocrine (Holsboer, 2001), and neuroanatomical components (Drevets et al., 2008) that contribute to the development of depression.

Brain regions involved in depression include limbic areas such as the anterior cingulate cortex, amygdala, prefrontal cortex, and hippocampus (Drevets et al., 2008). The hippocampus is a temporal lobe structure that is implicated in emotion and vulnerable to the effects of stress and depression (Drevets et al., 2008; McKinnon et al., 2009). Results from a meta-analysis show that individuals suffering from chronic (2+ years) or recurrent (2+ episodes) depression show smaller hippocampal volumes than healthy controls (McKinnon et al., 2009). Furthermore, hippocampal volume decreases with as length of time depressed increases, even after controlling for age (Sheline et al., 1999). In adults with depression, hippocampal volume is smallest in middle aged and older adults compared to younger adults (McKinnon et al., 2009), and though these data were not analyzed separately by sex, they suggest that normal levels of ovarian hormones in younger women may protect against hippocampal volume loss that occurs with depression. Interestingly, treatment with antidepressants seems to protect against hippocampal volume loss (Sheline et al., 2003; Lorenzetti et al., 2009), and premenopausal women are more likely than

men to show larger hippocampal volumes following successful antidepressant treatment (Vakili et al., 2000; Lorenzetti et al., 2009). The changes in hippocampal volume associated with the development and treatment of depression may be due to alterations in a number of factors such as changes in the number of neurons, glia, or neuropil, or cell death.

Adult hippocampal neurogenesis, the production of new neurons in adulthood, occurs in the dentate gyrus of the hippocampus of a variety of mammalian species, including humans and rodents (Eriksson et al., 1998; Altman and Das, 1965). The dentate gyrus is composed of the granule cell layer (GCL), an outer molecular layer, an inner region called the hilus, and a region between the GCL and hilus called the subgranular zone (see Figure 1). There are four stages of neurogenesis: cell proliferation, differentiation, migration, and survival. The timeline of new neuronal development differs between rats and mice (Snyder et al., 2009), and the timeline for rats is presented below. In rats, progenitor cells in the subgranular zone divide into daughter cells during a cell cycle lasting approximately 24 hours (Cameron and McKay, 2001). The daughter cells continue to proliferate or, in the following 1-5 days, start to differentiate into neurons or glia and migrate into the GCL. At 4-10 days of age, immature neurons begin extending axons through the hilus to the CA3 region of the hippocampus and dendrites to the molecular layer of the dentate gyrus (Hastings and Gould, 1999). The new neurons begin to make functional connections as they integrate into the existing hippocampal network, and at 2-3 weeks they begin to express mature neuronal markers (Brown et al., 2003). The amount of neurogenesis can be affected by altering any stage of neurogenesis. For example, chronic antidepressant treatment upregulates cell proliferation without independently affecting the differentiation, migration, or survival of new neurons, which results in a net increase in the number of new neurons (Malberg et al., 2000). Alternatively, treatment with testosterone increases the survival of new neurons

without independently affecting cell proliferation, and also results in a net increase in the number of new neurons (Spritzer and Galea, 2007).

Several animal models of depression capitalize on the previously mentioned link between stress and depression by using procedures such as exposure to chronic unpredictable stress and administration of chronic exogenous corticosterone (Airan et al., 2007; Wainwright et al., 2011; Brummelte and Galea, 2010; David et al., 2009). Both chronic unpredictable stress and chronic corticosterone increase depressive-like behaviours and reduce hippocampal cell proliferation, while treatment with antidepressants often attenuates depressive-like behaviour and upregulates cell proliferation in these models (Airan et al., 2007; David et al., 2009). Other therapies that help alleviate depression, such as exercise and electroconvulsive shocks, also increase cell proliferation in rodents (Olson et al., 2006; Malberg et al., 2000). Furthermore, ablation of neurogenesis can increase depressive-like behaviour on some behavioural measures (forced swim test, sucrose preference test: Snyder et al., 2011), and also blocks the ability of antidepressants to alleviate some depressive-like behaviours (forced swim test: Airan et al., 2007). It is also important to note that chronic, but not acute, antidepressant treatment upregulates cell proliferation, and it does so in a timeline that mirrors behavioural symptom alleviation in humans (Malberg et al., 2000).Taken together, these findings suggest that neurogenesis may be one mechanism through which antidepressants work to alleviate depressive-like behaviour.

There are important functional differences between the dorsal and ventral hippocampus. The ventral hippocampus is primarily involved in the regulation of stress and emotion, while the dorsal hippocampus is involved in memory and cognition (Bannerman et al., 2004; Fanselow and Dong, 2010). For example, lesion studies show a double dissociation between the dorsal and

ventral hippocampus: dorsal but not ventral lesions interfere with acquisition of spatial learning and memory tasks (Morris water maze: Moser et al., 1993; McHugh et al., 2004), while ventral but not dorsal lesions prevent or reduce anxiety-like behaviour (i.e. novelty suppressed feeding test: McHugh et al., 2004). Regional differences in anatomical connectivity and density of monoamine innervation further support this functional dissociation (Fanselow and Dong, 2010; Gage and Thompson, 1980). Based on the dissociation between the dorsal and ventral hippocampus, it is possible that the dorsal and ventral dentate gyrus exhibit different levels of cell proliferation in response to antidepressant treatment, and the three known studies conducted to date support this hypothesis (Airan et al., 2007; Banasr et al., 2006; Jayatissa et al., 2006). All three studies found that chronic antidepressant treatment increased cell proliferation in the ventral dentate gyrus, and one study (Jayatissa et al., 2006) also found an effect of SSRI treatment in the dorsal dentate gyrus. New neurons generated in the dorsal dentate gyrus following chronic SSRI treatment have also been shown to preferentially accumulate in the infrapyramidal, compared to the suprapyramidal, blade, which is not recruited during memory tasks (Satvat et al., 2012). Thus, further research is needed to elucidate the role of the ventral, but also the dorsal, dentate gyrus in antidepressant-induced increases in cell proliferation. However, it is plausible that the ability of antidepressants to alleviate depressive symptoms may be due to changes in cell proliferation in the ventral, and not the dorsal, dentate gyrus.

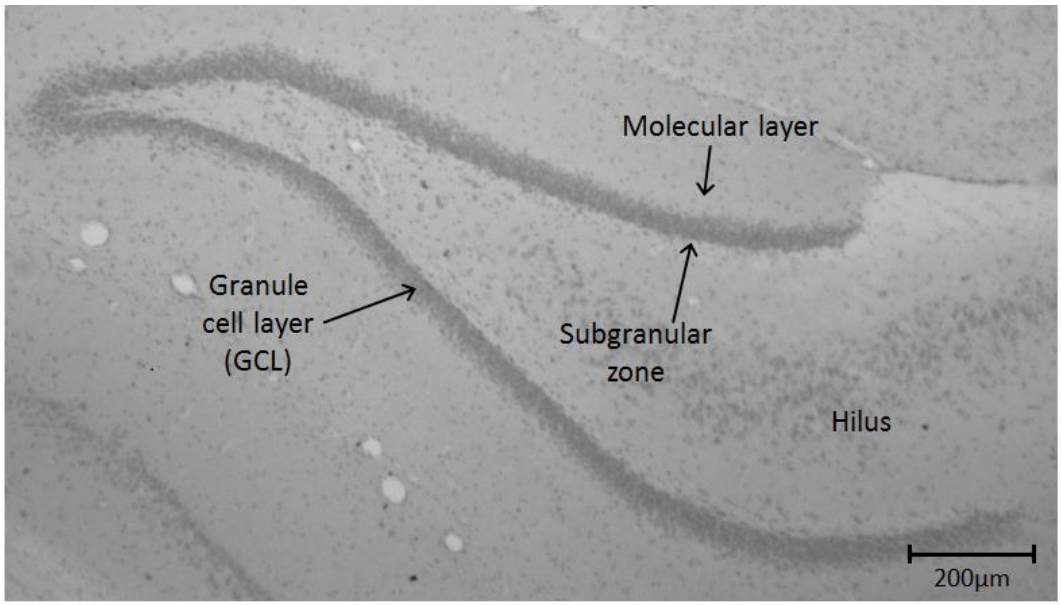

Figure 1. Photomicrograph of a coronal section of the dentate gyrus of the hippocampus from a female rat. Image viewed at 40X magnification.

The effect of ovarian hormones on antidepressant efficacy and hippocampal neurogenesis

Despite the previously discussed sex differences in incidence and treatment of depression, most research on animal models of depression has utilized male rodents. However, research using female rodents is warranted, as studies suggest that ovarian hormones may influence depressive-like behaviour and antidepressant response in females. Ovariectomizing (OVX) female rats, which eliminates ovarian hormones, can increase depressive-like behaviour on some behavioural tests (forced swim test: Estrada-Camarena et al., 2011; Frye & Wawrzycki, 2003). Consistent with human research, treating OVX females with medium or high doses of estradiol can alleviate depressive-like behaviour in these animals (forced swim test: Estrada-Camarena et al., 2011; Frye and Wawrzycki, 2003; Recambier-Carballo et al., 2012). However, TCA and SSRI antidepressants do not always alleviate depressive-like behaviour in OVX animals (Carrier and Kabbaj, 2012a); their effectiveness depends on many factors such as drug dosage, length of administration, time since OVX, age of subjects, and behavioural test

examined (forced swim test: Estrada-Camarena et al., 2011; Frye and Wawrzycki, 2003; Recambier-Carballo et al., 2012; sucrose preference test: Carrier and Kabbaj, 2012a). The inconsistent effect of antidepressants to alleviate depressive-like behaviour in OVX females potentially mirrors the human literature showing reduced antidepressant efficacy in post-menopausal women (Pae et al., 2009; Pinto-Meza et al., 2006; Grigoriadis et al., 2003), and suggests that lack of ovarian hormones may have a negative effect on antidepressant efficacy.

Ovarian hormones directly influence neurogenesis in the hippocampus of female rodents. The classical estrogen receptors (ERα and ERβ) have been localized in the hippocampus of adult female rats (Weiland et al., 1997), and proliferating cells in granule cell layer (GCL) of the dentate gyrus have been found to express ERα and ERβ in female rodents (Mazzucco et al., 2006). In line with these findings, both exogenous estradiol administration (Barha et al., 2009; Barker and Galea, 2008; Ormerod et al., 2003), and naturally occurring peaks in estradiol during the proestrous phase of the estrous cycle of rats (Tanapat et al., 1999) or during the breeding season of meadow voles (Galea and McEwen, 1999), are associated with alterations in cell proliferation in females. Specifically, short term (30 m) and repeated (15 d) exposure to estradiol increase call proliferation (Barha et al., 2009; Barker and Galea, 2008), while long term (48 h) exposure to estradiol suppresses cell proliferation in the dentate gyrus (Ormerod et al., 2003; Ormerod and Galea, 2001). Conversely, ovariectomizing adult female rats results in short term, but not long term, suppression of cell proliferation (Banasr et al., 2001; Tanapat et al., 1999; Green and Galea, 2008; Tanapat et al., 2005; Lagace et al., 2007).

Despite the associations between ovarian hormones and antidepressants, and ovarian hormones and neurogenesis, only one study has examined whether ovarian hormones modulate antidepressant-induced neurogenesis in females (Green and Galea, 2008). This study found that

chronic treatment with the TCA imipramine significantly increased cell proliferation in Sham, but not OVX, animals, suggesting that normal ovarian functioning may facilitate a neurogenic response to antidepressants in females. However, to date no study has compared the neurogenic response to different types of antidepressants in OVX compared to Sham females. The ovarian hormone modulation of antidepressant-induced hippocampal cell proliferation may be a novel mechanism regulating antidepressant efficacy, and more research in this area is warranted.

Objectives

The aim of the current experiment was to determine whether ovarian hormones interact with antidepressants to upregulate cell proliferation differentially in the dorsal and ventral regions of the dentate gyrus of female rats. To examine this, adult female rats were ovariectomized or sham ovariectomized, given one week to recover, and given daily injections with either the TCA imipramine (Trofanil), the SSRI fluoxetine (Prozac), or vehicle for 21 days prior to perfusion. We hypothesized that sham ovariectomized animals receiving chronic antidepressants would show a greater proliferative response than ovariectomized animals receiving antidepressants, and that antidepressants would upregulate cell proliferation to a greater extent in the ventral, compared to the dorsal, dentate gyrus.

METHODS

Animals

Thirty-three adult female Sprague-Dawley rats aged 148-160 d and weighing between 260-320 g were used in this study. Animals were bred and raised at the University of British Columbia (Vancouver, BC, Canada). Rats were double-housed in clear polyurethane cages (48 x 27 x 20 cm) containing a polyvinylchloride tube, paper towels, and sterile aspen chip bedding. Rats received ad libitum access to Purina rat chow (Lab Diet 5012; PMI Nutrition International, Brentwood, MO, USA) and tap water throughout the experiment. The room temperature was maintained at 21-22 ºC with lights operating on a 12 h light/dark cycle (lights on at 0700 h). Animals were handled five minutes per day for five days prior to the start of the experiment. The experiment was conducted in accordance with the Canadian Council for Animal Care ethical guidelines and was approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and suffering of animals.

Procedure

Surgery

At the start of the study animals were quasi-randomly assigned into groups based on body weight. Half of the animals then received either bilateral ovariectomies (OVX) or sham ovariectomies (Sham) using aseptic surgical technique while under isoflurane anesthesia (Baxter Corporation, Mississauga, ON, Canada). All animals were induced at a flow rate of 5% and maintained under anesthesia at a flow rate of 2.5-3%. Each animal received one subcutaneous (s.c.) 5 ml injection of lactated ringer's solution to maintain adequate fluid levels (Hospira, Montreal, QC, Canada), one s.c. 5 mg/kg injection of the non-steroidal anti-inflammatory

analgesic Anafen (MERIAL Canada Inc., Baie D'Urfé, QC, Canada), and one s.c. 4 mg/kg injection of the local anesthetic Bupivacaine divided between both incision sites (Hospira, Montreal, QC, Canada). OVX animals had both ovaries removed through small bilateral flank incisions, and Sham animals underwent the same procedure but ovaries were left intact. Immediately after surgery the topical antibacterial ointment Flamazine was applied to the exterior of the incisions (Smith & Nephew, Saint Laurent, QC, Canada) and animals were monitored and kept warm until fully recovered from the anesthetic. Prior to drug treatment animals were given seven days to recover from surgery, during which they were individually housed and monitored and weighed daily.

Drug treatment

After recovery from surgery, OVX and Sham animals received 21 days of intraperitoneal injections with either 15 mg/kg of the tricyclic antidepressant imipramine (IMI; Sigma-Aldrich, Saint Louis, MO, USA); 10 mg/kg of the SSRI antidepressant fluoxetine (FLX; Sequoia Research Products Ltd., Pangbourne, UK); or 1 ml/kg of the sterile water vehicle (water; Hospira Healthcare Corp., Montreal, QC, Canada), for a total of six groups (n = 5-6 per group). The volume of all injections was 1 ml/kg, and animals were weighed every other day to ensure that injection volumes were based on current body weights. A dose of 15 mg/kg of imipramine was chosen because it upregulates cell proliferation in female rats (Green and Galea, 2008). Doses of both 10 mg/kg and 20 mg/kg of fluoxetine have been shown to upregulate cell proliferation in females (Lagace et al., 2007; Airan et al., 2007); therefore, we used the lower dose of 10 mg/kg because it has also been shown to upregulate cell proliferation in male rats (Malberg and Duman, 2003) and female mice (Lagace et al., 2007). All injections occurred between 0830 and 1030 h.

Perfusion

Twenty-four hours after the last drug injection, animals were weighed, given an overdose of sodium pentobarbital (Euthanyl; Bimeda-MTC, Cambridge, ON, Canada), and perfused transcardially using 60 ml of 0.1 M phosphate buffered saline (PBS) followed by 120 ml of 4% paraformaldehyde. Brains were extracted, post-fixed using 4% paraformaldehyde at 4 ºC for 24 h, and transferred to 30% sucrose in 0.1 M PBS until sectioning. To further elucidate the relationship between ovarian and adrenal hormones in antidepressant efficacy, adrenal glands were also extracted and weighed.

Estrous cycle determination

Research suggests that antidepressants have an effect on the length of the estrous cycle in female rats (Maswood and Uphouse, 1992; Uphouse et al., 2006). Furthermore, estrous cycle phase can influence hippocampal cell proliferation, as rats in proestrus (when estradiol levels are highest) have significantly more cell proliferation than rats in any other stage (Tanapat et al., 1999). Therefore, throughout the 21 days of drug treatment, and also on perfusion day, vaginal cells of all animals were collected by lavage, transferred to microscope slides, and stained with cresyl violet. The lavage slides of Sham animals were then analyzed under a 10X objective lens to determine the number of days each animal was in proestrus throughout the 21 days of drug treatment, as well as the number of animals in proestrus on perfusion day. Animals were found to be in proestrus when the majority of cells (at least 70%) were nucleated epithelial cells. See Figure 2 for experimental timeline.

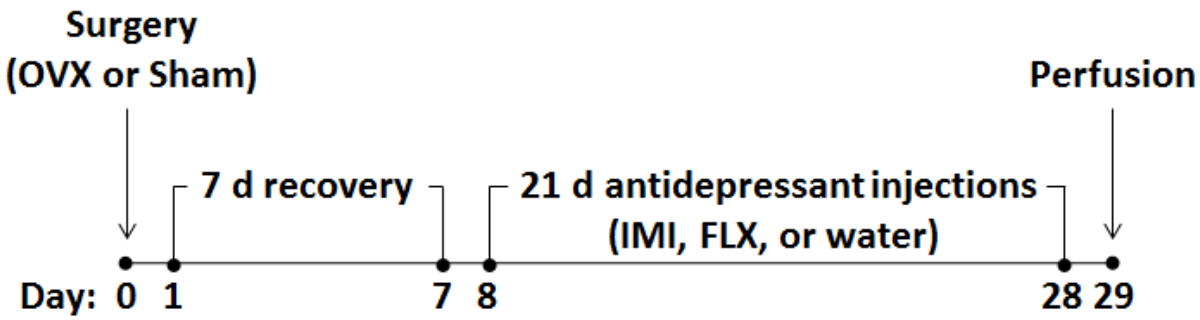

Figure 2. Experimental timeline. OVX: ovariectomy. Sham: sham ovariectomy. IMI: imipramine. FLX: fluoxetine.

Immunohistochemistry

Brains were sectioned along the entire rostral-caudal extent of the hippocampus into ten series of 40 µm coronal sections using a Leica SM2000R freezing sliding microtome (Leica Microsystems, Richmond Hill, ON, Canada). Series were then stained for two endogenous markers, Ki67 and doublecortin (DCX). Ki67 is a nuclear protein expressed during all phases of the cell cycle except G_0 and a small part of G_1, and it labels dividing cells undergoing proliferation in the previous 24 h (Kee et al., 2002). DCX is a microtubule-associated cytoplasmic protein, which labels immature neurons in the previous 1-21 d (Brown et al., 2003).

Ki67

In both staining protocols tissue was rinsed three to five times in 0.1 M PBS for 10 m per rinse between each step. Tissue was pretreated with 0.6% hydrogen peroxide for 30 m at room temperature to block endogenous peroxidase activity, then incubated in primary antibody solution consisting of 1:1000 monoclonal rabbit anti-Ki67 (Vector Laboratories, Burlingame, CA, USA), 0.04% Triton-X, and 3% normal goat serum, dissolved in 0.1 M PBS, at 4 ºC for 24 h. The tissue was then incubated in secondary antibody solution containing 1:500 biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS, at 4 ºC for 24 h,

followed by incubation in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 4 h at room temperature. Lastly, Ki67-labeled cells were visualized using 0.02% diaminobenzidine (Sigma-Aldrich, Oakville, ON, Canada) with 0.0003% hydrogen peroxide in sodium acetate buffer for approximately 10 m. Tissue was then mounted on glass slides, counterstained using cresyl violet, dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, New Jersey, USA).

Doublecortin

A second series of tissue was pretreated with 0.6% hydrogen peroxide for 30 m at room temperature; incubated in primary antibody solution consisting of 1:1000 polyclonal goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.04% Triton-X, and 3% normal rabbit serum, dissolved in 0.1 M PBS, at 4 ºC for 24 h; and incubated in secondary antibody solution containing 1:500 biotinylated rabbit anti-goat (Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS, at 4 ºC for 24 h. Tissue was then incubated in avidin-biotin complex for 4 h at room temperature, and doublecortin-labeled cells were visualized using 0.02% diaminobenzidine with 0.0003% hydrogen peroxide and 2.5% nickel, in sodium acetate buffer for approximately 10 m. Once staining was complete, tissue was mounted on glass slides, dehydrated, and coverslipped with Permount.

Cell counting

Cell counting was conducted by a researcher blind to treatment conditions. Ki67- and DCX-labeled cells were counted in every 10^{th} section throughout the extent of the granule cell layer (GCL), and an estimate of the total number of cells in the entire GCL was obtained by multiplying the total number of cells counted by ten (Barha et al., 2009; Barker and Galea 2008).

Because there are potential functional differences between the dorsal and ventral hippocampus (Bannerman et al., 2004), separate cell counts were obtained for the dorsal and ventral GCL, with all slices rostral to -4.8 mm from bregma considered dorsal and all slices caudal to -4.8 mm from bregma considered ventral (Banasr et al., 2006). Cells were counted using an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) under a 100X oil immersion objective lens.

To determine whether differences exist in the maturational stage of DCX-labeled immature neurons, 100 DCX-labeled cells (30 dorsal and 70 ventral) were selected arbitrarily from all sections counted per brain. Cells were classified into one of three stages, from least to most mature: absent or short processes (< 10 μm); medium processes (> 10 μm) with zero or one branch points reaching the GCL; or long processes with multiple branch points reaching the GCL or molecular layer (Plumpe et al., 2007; Epp et al., 2011). See Figure 3 for representative photomicrographs of Ki67- and DCX-labeled cells.

The area of the GCL of all sections counted was measured using the image analysis software program ImageJ (Bethesda, MD, USA). For each animal, the summed areas were then multiplied by the distance between sections (400 μm) using Cavalieri's principle (Gundersen & Jensen, 1987) to provide an estimate of the volume of the GCL. Separate volume measurements were calculated for the dorsal and ventral GCL.

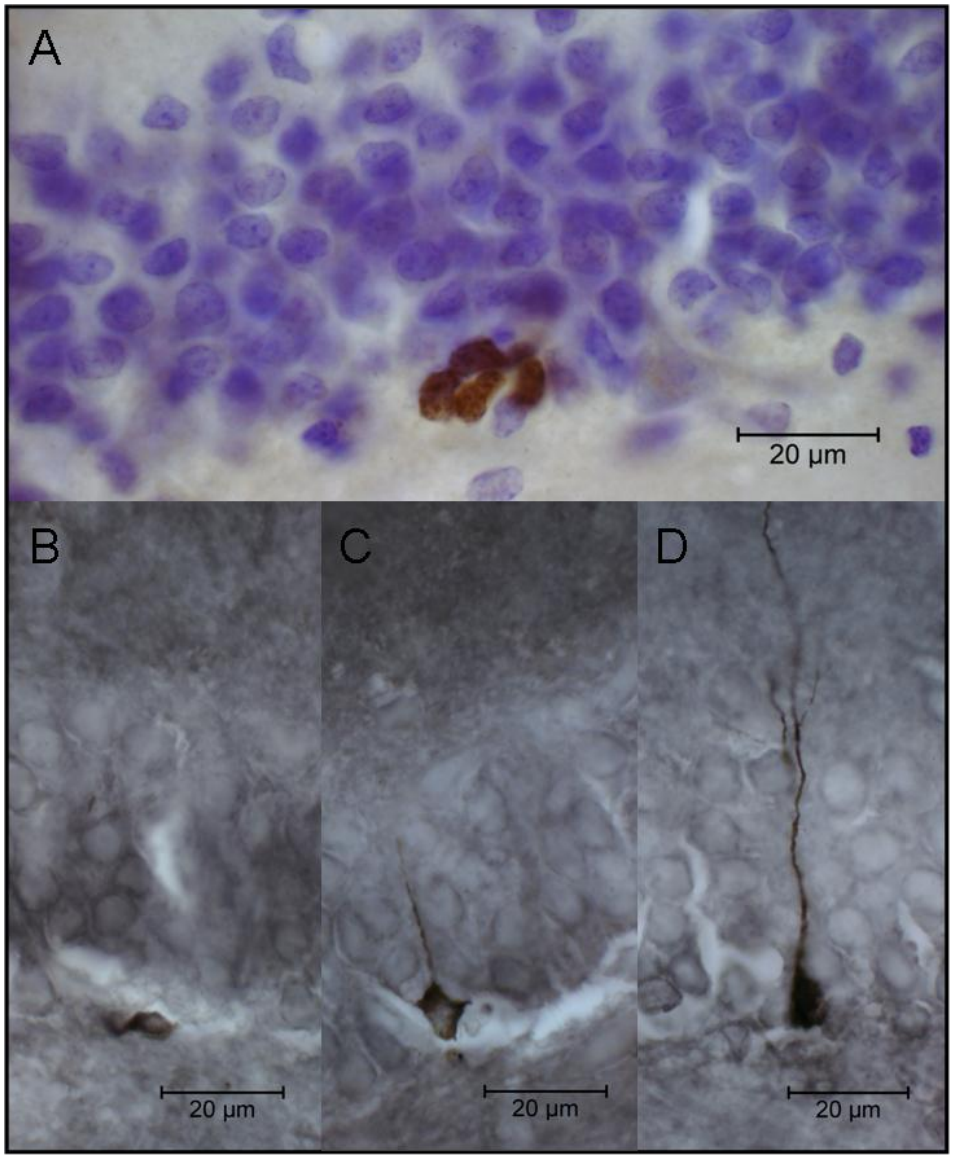

Figure 3. Representative photomicrographs of Ki67- and doublecortin-labeled cells in the granule cell layer (GCL). **A)** A clump of Ki67-labeled cells in the subgranular zone of the GCL. **B)** Doublecortin-labeled cell in the subgranular zone with a short process, **C)** a medium process reaching into the GCL, and **D)** a long, branched process reaching into the molecular layer. All images viewed at 1000X magnification.

Data analyses

Body weight was analyzed using a repeated-measures ANOVA with day (1-21) as a within-subjects factor and surgery (Sham, OVX) and drug (water, IMI, FLX) as between-

subjects factors. Volume of the GCL and total number of Ki67- and DCX-labeled cells were examined using repeated-measures ANOVAs, with region (dorsal, ventral) as a within-subjects factor and surgery and drug as between-subjects factors. Estrous cycle (proestrus, not proestrus) was used as a covariate in the Ki67 analyses. Repeated-measures ANOVAs were used to examine stage of maturation of DCX-labeled cells, with region (dorsal, ventral) and stage (short processes, medium processes, long processes) as the within-subjects factors and surgery and drug as the between-subjects factors. Adrenal to body weight ratios were analyzed using a two-way ANOVA with surgery and drug as between-subjects factors, and Pearson product-moment correlations were calculated to correlate adrenal to body weight ratios with both Ki67-labeled cells in the dorsal and ventral GCL and DCX-labeled cells in the dorsal and ventral GCL. Number of days that Sham animals spent in proestrus was calculated using a one-way ANOVA with drug as the between-subjects factor. All statistical analyses were set at $\alpha = 0.05$. All a priori comparisons were subjected to Bonferroni correction. All post-hoc tests were conducted using Newman-Keuls. The software program Statistica (StatSoft Inc., Tulsa, OK, USA) was used to conduct all analyses.

RESULTS

Ovariectomy increased, and chronic antidepressant treatment decreased, body weight compared to water controls

Groups did not differ in body weight at the start of the study, $F(5,27) = 0.98$, $p = 0.45$. Figure 4 presents body weight throughout the 21 days of drug injections. As expected, there was a significant day by surgery interaction, $F(10,270) = 4.62$, $p < 0.001$, with post-hoc tests showing that OVX animals gained significantly more weight than Sham animals from the fifth day of drug injections onward (all p's < 0.042). Furthermore, there was a significant day by drug interaction, $F(20,270) = 3.60$, $p < 0.001$, where animals treated with IMI and FLX lost significantly more weight than those treated with water, from the third day of drug injections onward (all p's < 0.015). There were also significant main effects of surgery, $F(1, 27) = 22.05$, $p < 0.001$, and drug, $F(2,27) = 4.39$, $p = 0.022$.

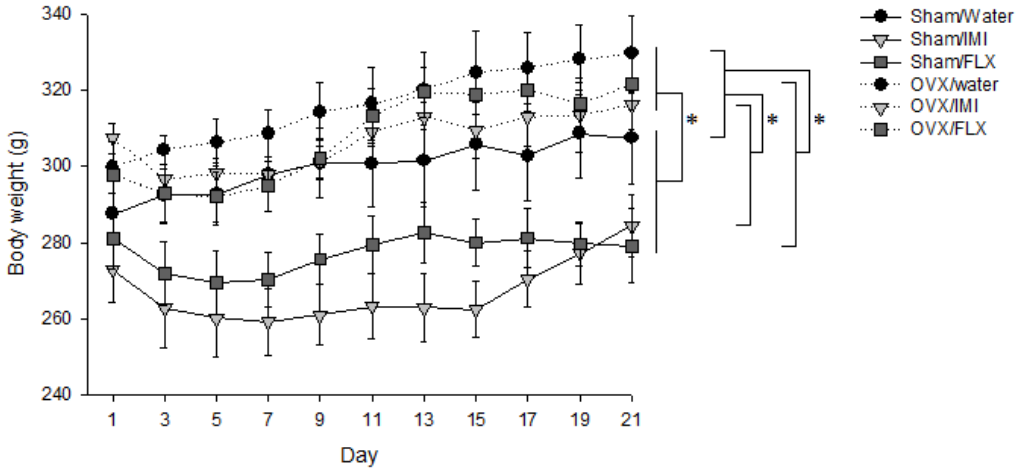

Figure 4. Mean (± SEM) body weight (g) of female rats during 21 days of antidepressant treatment. OVX compared to Sham animals gained more weight from day 5 of drug injections onward (all p's < 0.042). Animals receiving imipramine and fluoxetine compared to water lost more weight from day 3 of drug injections onward (all p's < 0.015). * indicates significantly different from each other during time periods specified above.

The volume of the dorsal and ventral GCL did not differ between groups, but the ventral GCL was larger.

As seen in Table 1, neither surgery ($p = 0.39$) nor drug treatment ($p = 0.12$) altered the volume of the dorsal or ventral GCL, and there were no significant interactions (all p's > 0.66). As expected, there was a main effect of region, with the ventral GCL being larger than the dorsal GCL, $F(1,27) = 182.41$, $p < 0.001$. Because GCL volumes did not differ between groups, the total number of Ki67- and DCX-labeled cells was used for all immunohistochemical analyses.

Table 1. Mean volume (± SEM) of the dorsal and ventral granule cell layer (GCL) across groups.

	GCL volume (mm^3)	
	Dorsal	Ventral
Sham		
Water	1.18 ± 0.07	2.03 ± 0.15
Imipramine	1.06 ± 0.04	2.04 ± 0.14
Fluoxetine	1.07 ± 0.08	1.91 ± 0.12
Ovariectomized		
Water	1.21 ± 0.08	2.24 ± 0.20
Imipramine	1.20 ± 0.07	2.10 ± 0.14
Fluoxetine	1.06 ± 0.03	1.86 ± 0.15

Dorsal and ventral granule cell layer (GCL) volumes did not differ between groups. As expected, the ventral GCL was larger than the dorsal GCL ($p < 0.001$).

Chronic imipramine treatment increased cell proliferation in the ventral GCL of Sham animals, and chronic fluoxetine treatment increased cell proliferation in the dorsal GCL of OVX animals.

Figure 5 presents the total number of Ki67-labeled cells in both the dorsal (A) and ventral (B) GCL. There was a significant main effect of region, $F(1,23) = 48.11$, $p < 0.001$, with significantly more Ki67-labeled cells in the ventral compared to the dorsal GCL. A main effect

of surgery was also observed, $F(1,23) = 6.09$, $p = 0.021$, with OVX animals having significantly more Ki67-labeled cells than Sham animals. There was also a main effect of drug, $F(2,23) = 3.34$, $p = 0.050$, and a trend toward a region by drug interaction, $F(2,23) = 3.22$, $p = 0.059$. No other significant effects were found (all p's > 0.10). There was no significant effect of the covariate, proestrous, on total number of Ki67-labeled cells ($p = 0.22$), potentially due to the small number of animals in proestrus on perfusion day (n = 3 out of 16).

Based on previous published reports, a priori we expected antidepressants to increase cell proliferation differentially depending on both ovarian hormone status (Green and Galea, 2008) and region of the dentate gyrus examined (Banasr et al., 2006). A priori comparisons revealed that Sham/IMI animals had a significantly greater total number of Ki67-labeled cells compared to Sham/water controls in the ventral GCL ($p = 0.003$; see Figure 5B). In OVX animals neither imipramine nor fluoxetine increased cell proliferation compared to OVX/water controls in the ventral GCL (both p's > 0.43). OVX/water compared to Sham/water controls had a strong trend for higher baseline levels of cell proliferation in the ventral GCL ($p = 0.013$; Bonferroni correction significant p-value is $p = 0.01$). Interestingly, in the dorsal GCL of OVX animals, FLX significantly increased the total number of Ki67-labeled cells compared to OVX/water controls ($p = 0.009$; see Figure 5A). There were no other significant group differences in the dorsal GCL (all p's > 0.072).

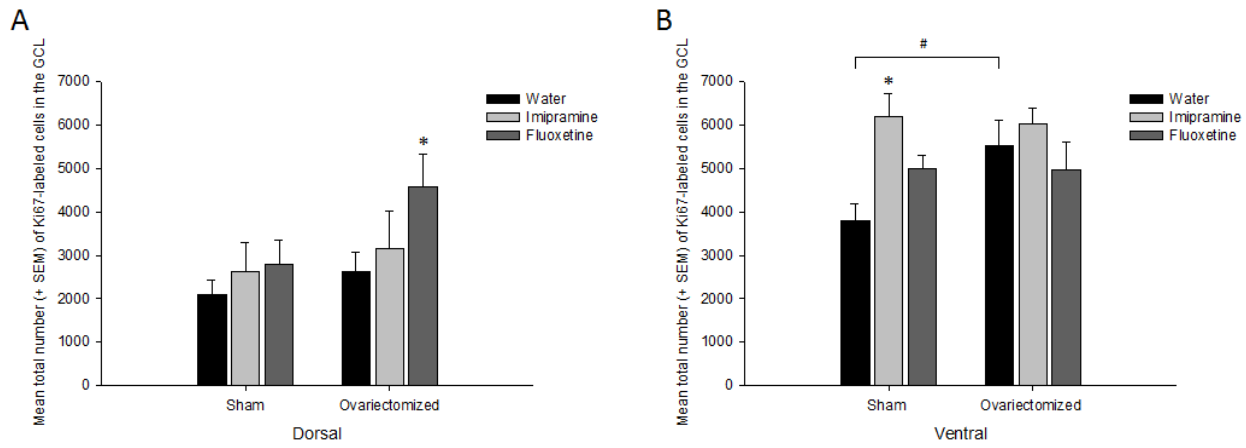

Figure 5. Mean total number (+ SEM) of Ki67-labeled cells in the dorsal and ventral granule cell layer (GCL) across groups. **A)** OVX animals receiving fluoxetine showed increased cell proliferation in the dorsal GCL ($p = 0.009$). **B)** Sham animals receiving imipramine showed increased cell proliferation in the ventral GCL ($p = 0.003$). OVX/water controls tended to show increased cell proliferation compared to Sham/water controls ($p = 0.013$). * indicates significantly different from respective control group. # indicates tendency to differ from each other.

Imipramine increased the number of immature neurons in the ventral GCL of OVX animals, and OVX animals receiving imipramine also had more 'mature' doublecortin-labeled cells with long/branched and medium processes in the ventral GCL.

Figure 6 presents the total number of DCX-labeled cells in both the dorsal (A) and ventral (B) GCL. There was a significant main effect of region, $F(1,23) = 84.25$, $p < 0.001$, with significantly more immature neurons in the ventral compared to the dorsal GCL. There was also a significant main effect of drug, $F(1,23) = 4.67$, $p = 0.020$, but no other significant main or interaction effects (all p's > 0.24). A priori we expected antidepressants to increase immature neurons differentially in a surgery-specific and region-specific manner (Green and Galea, 2008; Banasr et al., 2006). There were no significant drug effects in the dorsal GCL (all p's > 0.14; see Figure 6A). In the ventral GCL, a priori comparisons revealed that OVX/IMI animals had a significantly greater number of DCX-labeled cells compared to both OVX/water and OVX/FLX

animals (both p's < 0.005; see Figure 6B). There were no other significant drug effects in the ventral GCL (all p's > 0.058).

Maturational stage of DCX-labeled cells was also examined. We first examined the percentage of DCX-labeled cells at each maturational stage. As seen in Table 2, there was a main effect of maturational stage, $F(2,54) = 284.00$, $p < 0.001$, and post-hoc analyses revealed that all groups had significantly more DCX-labeled cells with long/branched compared to medium, and medium compared to absent/short, processes (all p's < 0.001). There were no other significant main effects (all p's > 0.17) and no significant interaction effects (all p's > 0.28).

We next multiplied the percentage of DCX-labeled cells at each maturational stage by the total number of DCX-labeled cells in the dorsal and ventral GCL. As seen in Figure 7, there were significant region by stage, $F(2,46) = 35.93$, $p < 0.001$, and stage by drug, $F(4,46)=3.28$, $p = 0.019$, interactions. There were also main effects of maturational stage, $F(2,46) = 255.95$, $p < 0.001$, region, $F(1,23) = 84.12$, $p < 0.001$, and drug, $F(2,23) = 4.69$, $p = 0.012$. Post-hoc analyses revealed that there were significantly more DCX-labeled cells with long/branched, compared to medium and absent/short, processes, in both the dorsal and ventral GCL (all p's < 0.001; see Figures 7A and 7B). In the ventral GCL there were also significantly more DCX-labeled cells with medium, compared to absent/short, processes ($p < 0.001$; see Figure 7B). Based on the increased total number of DCX-labeled cells in the ventral GCL of OVX/IMI animals, a priori we hypothesized that OVX/IMI animals would show differences in maturational stage of DCX-labeled cells in the ventral GCL. A priori comparisons revealed that OVX/IMI animals showed a strong trend for a greater number of cells with long/branched processes in the ventral GCL compared to all other groups (all p's < 0.013; Bonferroni correction significant p-value is p =

0.01). OVX/IMI animals also had a significantly greater number of cells with medium processes compared to OVX/water controls ($p = 0.002$).

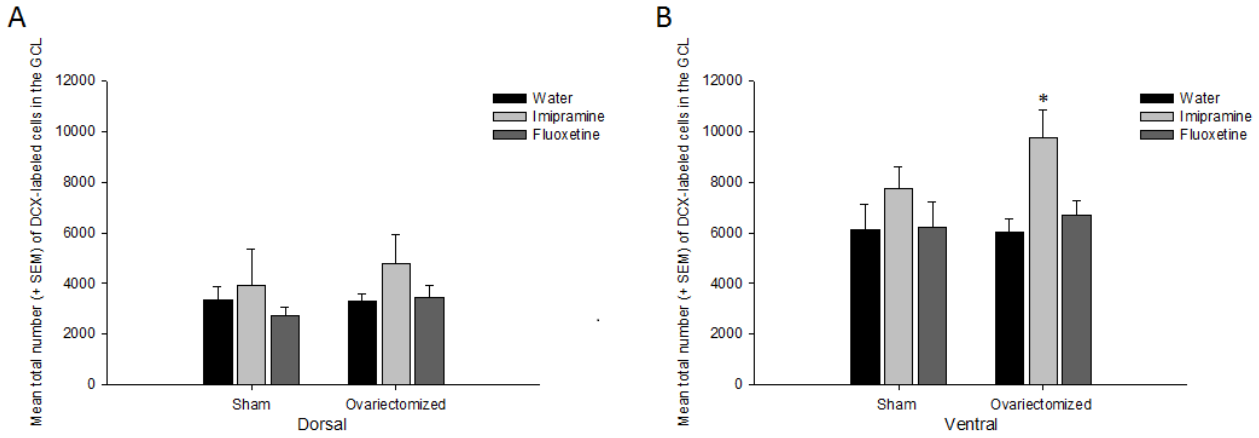

Figure 6. Mean total number (+ SEM) of doublecortin-labeled cells in the dorsal and ventral granule cell layer (GCL) across groups. **A)** The number of immature neurons in the dorsal GCL did not differ between groups (all p's > 0.14). **B)** Imipramine increased the number of immature neurons in the ventral GCL of OVX animals ($p < 0.001$). * indicates significantly different from OVX/water and OVX/FLX groups.

Table 2. Mean percentage (± SEM) of doublecortin-labeled cells at different maturational stages in the dorsal and ventral granule cell layer (GCL) across groups.

	Dorsal GCL			Ventral GCL		
	Length of processes					
	Short	Medium	Long	Short	Medium	Long
Sham						
Water	11.61 ± 2.23	28.33 ± 2.55	60.06 ± 4.42	13.31 ± 1.96	25.48 ± 2.82	61.17 ± 3.92
IMI	12.00 ± 2.71	28.07 ± 4.05	60.00 ± 6.41	7.43 ± 1.94	30.86 ± 2.50	61.74 ± 4.25
FLX	10.00 ± 1.05	27.40 ± 2.90	62.60 ± 2.21	10.00 ± 1.50	31.14 ± 4.20	58.86 ± 2.76
OVX						
Water	5.50 ± 1.40	28.83 ± 6.82	65.56 ± 6.65	6.43 ± 1.32	25.24 ± 2.73	68.33 ± 2.62
IMI	13.89 ± 3.38	36.17 ± 5.49	50.00 ± 6.32	9.29 ± 2.08	32.40 ± 2.56	58.36 ± 3.64
FLX	9.40 ± 3.39	27.33 ± 5.72	63.33 ± 6.41	8.54 ± 2.07	29.71 ± 2.76	61.71 ± 4.64

In both the dorsal and ventral granule cell layer of all groups there were more cells with long/branched compared to medium, and medium compared to absent/short, processes (all p's < 0.001). IMI: imipramine. FLX: fluoxetine.

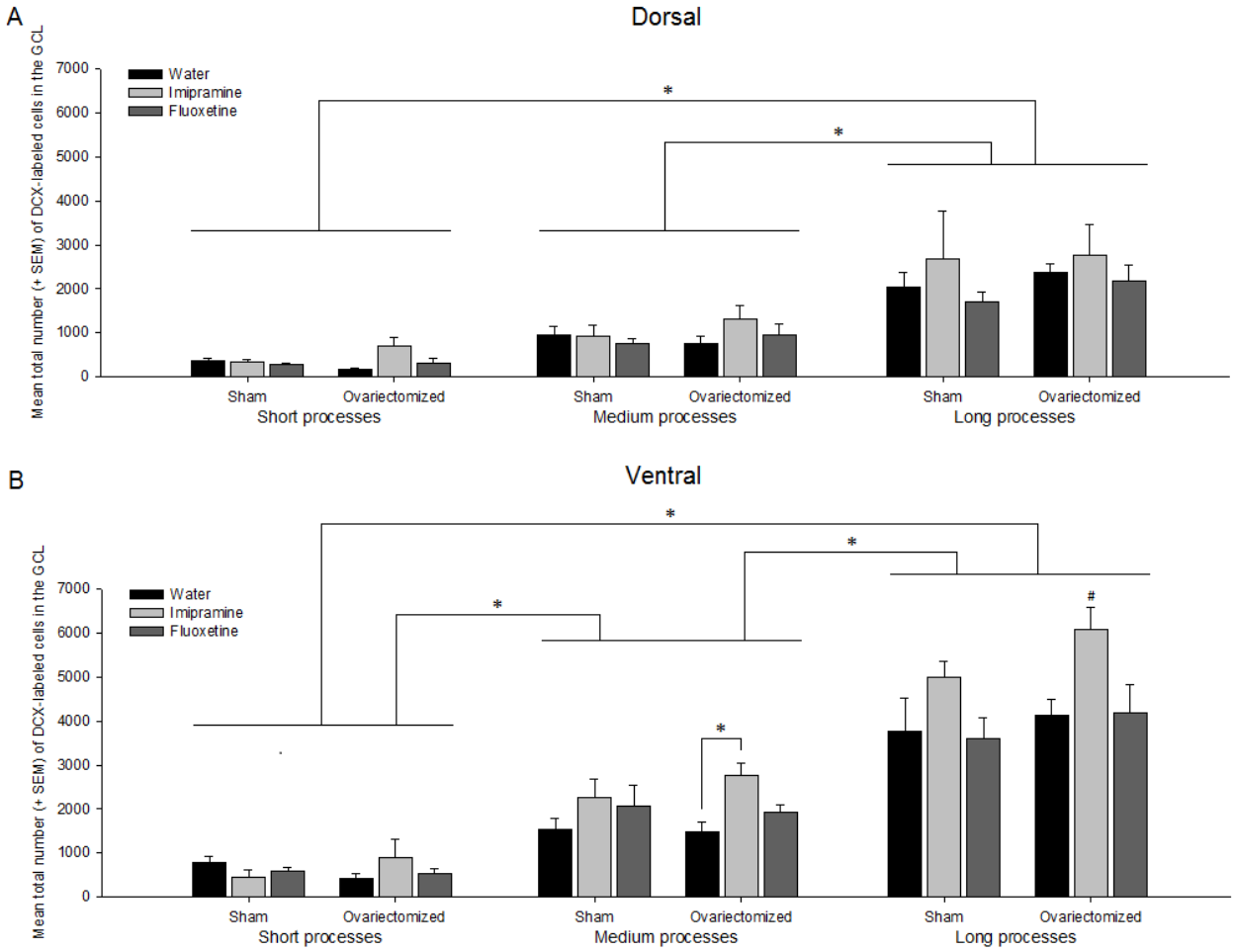

Figure 7. Mean total number (+ SEM) of doublecortin-labeled cells at different maturational stages in the dorsal and ventral granule cell layer (GCL) across groups. **A, B)** There were more DCX-labeled cells with long/branched, compared to medium and absent/short, processes (dorsal, ventral) and medium, compared to absent/short, processes (ventral). **B)** In the ventral GCL, the OVX/IMI group tended to have more neurons with long/branched processes (all p's < 0.013) and had significantly more neurons with medium processes (p = 0.002). * indicates significantly different from each other. # indicates tendency to differ from all other groups.

Compared to Sham/water controls, OVX/water controls had decreased adrenal to body weight ratios that were restored following chronic treatment with imipramine and fluoxetine.

Figure 8 presents adrenal to body weight ratios across groups. There was a significant main effect of drug, $F(1,27) = 12.21$, $p < 0.001$, and a significant surgery by drug interaction,

$F(2,27) = 6.048$, $p = 0.007$, but no significant main effect of surgery ($p = 0.09$). Post-hoc tests found that OVX/water controls had significantly smaller adrenal to body weight ratios than Sham/water controls ($p = 0.008$). Treatment with IMI and FLX significantly increased adrenal ratios in OVX animals compared to water controls (both p's < 0.007), but there was no effect of antidepressants to alter adrenal ratios in Sham animals (both p's > 0.09). We also examined whether adrenal to body weight ratios were correlated with total number of Ki67- or DCX-labeled cells. Adrenal ratios were not significantly correlated with either Ki67-labeled cells in the dorsal or ventral GCL (both p's > 0.44) or DCX-labeled cells in the dorsal or ventral GCL (both p's > 0.10).

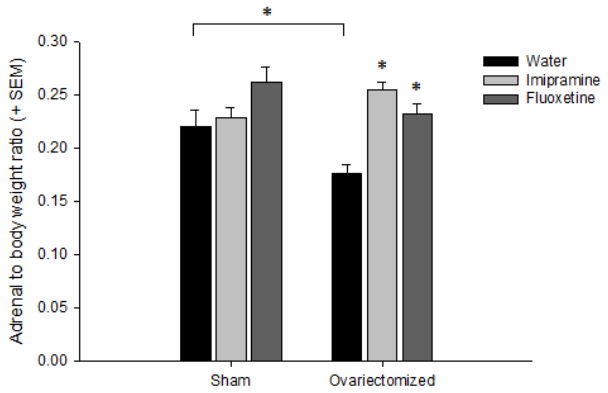

Figure 8. Adrenal to body weight ratios (+ SEM) across groups. OVX/water compared to Sham/water controls had smaller adrenal to body weight ratios ($p = 0.008$) that were restored following treatment with imipramine and fluoxetine (both p's < 0.007). * indicates significantly different from OVX/water controls.

Compared to Sham/water controls, Sham animals receiving chronic treatment with imipramine and fluoxetine spent significantly fewer days in the proestrus phase of the estrous cycle

Figure 9 presents the total number of days Sham animals spent in the proestrus phase of the estrous cycle throughout the 21 days of antidepressant injections. There was a significant

main effect of drug, $F(2,11) = 7.37$, $p = 0.009$, with post-hoc tests revealing that Sham/IMI and Sham/FLX animals spent significantly fewer days in proestrus compared to Sham/water controls (both p's < 0.014).

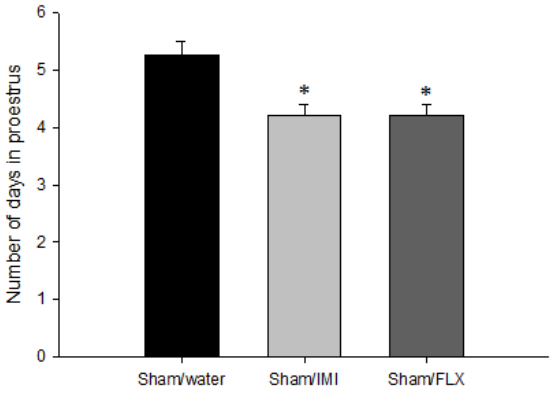

Figure 9. Number of days Sham animals spent in the proestrus phase of the estrous cycle during 21 days of antidepressant treatment. Sham/IMI and Sham/FLX animals spent significantly fewer days in proestrus compared to Sham/water controls (both p's < 0.014). * indicates significantly different from Sham/water controls.

DISCUSSION

The current study is the first to demonstrate that ovarian hormone levels modulate the ability of chronic antidepressant treatment to upregulate hippocampal cell proliferation in females in a drug- and region-specific manner. Specifically, chronic treatment with the TCA imipramine significantly increased cell proliferation (Ki67-labeled cells) in the ventral dentate gyrus of Sham, but not OVX, animals. Conversely, chronic treatment with the SSRI fluoxetine significantly increased cell proliferation in the dorsal dentate gyrus of OVX, but not Sham, animals. These findings suggest that normal ovarian functioning facilitates a neurogenic response to imipramine in the ventral dentate gyrus, which may be related to the antidepressant properties of this drug. Conversely, the fluoxetine-induced increase in cell proliferation in the dorsal dentate gyrus may relate to the ability of fluoxetine to alleviate the cognitive decline often seen with depression. We also found significantly more immature neurons (DCX-labeled cells) in the ventral dentate gyrus of OVX animals receiving imipramine, which may be related to differences in the maturation rates of immature neurons in OVX/IMI animals. OVX/water compared to Sham/water controls had significantly decreased adrenal to body weight ratios that were restored following chronic treatment with imipramine and fluoxetine, indicating that antidepressants help regulate HPA axis function in OVX animals. Lastly, Sham animals receiving chronic treatment with imipramine and fluoxetine spent significantly less days in the proestrus phase of the estrous cycle compared to Sham/water controls, which is consistent with previous research and suggests that chronic treatment with imipramine and fluoxetine increases the length of the estrous cycle in females (Maswood and Uphouse, 1992; Uphouse et al., 2006).

Taken together, our findings demonstrate that ovarian hormones modulate the effect of antidepressants on both HPA axis function and hippocampal neurogenesis.

Chronic imipramine treatment increased cell proliferation in the ventral dentate gyrus of Sham, but not OVX, female rats

In the present study, chronic treatment with imipramine increased cell proliferation (Ki67-labeled cells) in the ventral dentate gyrus of Sham, but not OVX, animals. Our findings are consistent with behavioural studies (Carrier and Kabbaj, 2012a; Green et al., 2009), as well as with the only study to date that has examined the ovarian hormone regulation of antidepressant-induced cell proliferation (Green and Galea, 2008). Green and Galea (2008) found that chronic treatment with imipramine increased cell proliferation in the dentate gyrus of Sham, but not OVX, females; however, they did not examine cell proliferation separately in the dorsal and ventral dentate gyrus. The important role of the ventral hippocampus in stress and emotion (Bannerman et al., 2004) suggests that cell proliferation in the ventral dentate gyrus may contribute directly to antidepressant efficacy. The current results suggest that in females, normal cycling ovarian hormones facilitate the neurogenic response to imipramine in the ventral dentate gyrus.

Behavioural studies support the idea that the antidepressant effect of imipramine is related to ovarian hormone levels, with normal levels of ovarian hormones associated with alleviation of depressive-like behaviour. Research has shown that chronic imipramine treatment alleviated depressive-like behaviour in intact females (forced swim test: Airan et al., 2007), but failed to alleviate depressive-like behaviour in OVX females (sucrose preference: Carrier and Kabbaj, 2012a; Green et al., 2009). Following antidepressant treatment, women with normal,

compared to low, ovarian hormone functioning showed a greater decline in depressive symptoms (Pae et al., 2009; Pinto-Meza et al., 2006; Grigoriadis et al., 2003), suggesting that normal ovarian hormone functioning results in a better antidepressant response. Interestingly, hypogonadal males compared to females differ in their behavioural and neurogenic response to imipramine. While OVX females failed to show a behavioural response to chronic imipramine treatment, gonadectomized males showed both alleviation of depressive-like behaviour and upregulation of cell proliferation following chronic treatment with imipramine (Carrier and Kabbaj, 2012b). Thus, the current study demonstrates that low levels of ovarian hormones decrease hippocampal responsivity to imipramine in females. This finding provides insight into a potential mechanism of action underlying a lack of antidepressant efficacy in depressed women with low levels of ovarian hormones.

In the current study, chronic treatment with fluoxetine did not increase cell proliferation in the ventral dentate gyrus of Sham or OVX animals. The current study used a dose of 10 mg/kg, and it is possible that this dose was too low to have a significant effect on cell proliferation in the ventral dentate gyrus. This is partially consistent with research in female rats that showed that chronic treatment with a low dose (5 mg/kg) of fluoxetine had no effect on overall cell proliferation (Hodes et al., 2009). However, chronic treatment with a very high dose (20 mg/kg) increased cell proliferation in the ventral dentate gyrus (Airan et al., 2007). In the interest of using the lowest possible effective dose, the current study chose to use an intermediate dose of 10 mg/kg because this dose upregulated cell proliferation in female mice (Lagace et al., 2007) and male rats (Malberg et al., 2003). The lack of effect of fluoxetine to increase cell proliferation in the ventral dentate gyrus of Sham or OVX females may demonstrate that females

require a higher dose than males to show upregulation of cell proliferation, highlighting the importance of considering both sex and dose when treating with antidepressants.

It is also possible that in females, imipramine works to alleviate depressive-like behaviour through upregulation of hippocampal cell proliferation while fluoxetine alleviates depressive-like behaviour largely through neurogenesis-independent mechanisms (i.e. synaptic plasticity: Bessa et al., 2009). Consistent with the current study, a post-mortem study in humans found that depressed subjects treated with chronic TCA, but not SSRI, antidepressants had significantly more proliferating (Ki67-labeled) cells in the anterior dentate gyrus, which corresponds to the ventral dentate gyrus in rodents (Boldrini et al., 2009). The subjects receiving TCAs also had increased dentate gyrus volumes that may have been due to the increase in cell proliferation. However, the study was underpowered to examine males and females separately, so it is currently unknown whether there is an effect of SSRIs on cell proliferation in one sex but not the other.

Chronic fluoxetine treatment increased cell proliferation in the dorsal dentate gyrus of OVX, but not Sham, female rats

Interestingly, the current study found that chronic treatment with fluoxetine increased cell proliferation in the dorsal dentate gyrus of OVX animals only. This finding is partially consistent with past research showing that chronic SSRI treatment upregulates cell proliferation in the dorsal dentate gyrus of male rats (Jayatissa et al., 2006), and also upregulates the survival of proliferating cells in the infrapyramidal blade of the dorsal dentate gyrus (Satvat et al., 2012). The dorsal hippocampus is primarily involved in memory and cognition (Bannerman et al., 2004), though recent research suggests that the infrapyramidal blade of the dorsal dentate gyrus

may actually serve a broader role in cognition and mood (Satvat et al., 2012). Thus, the fluoxetine-induced increase in cell proliferation in the dorsal dentate gyrus OVX animals may relate to the ability of fluoxetine to alleviate cognitive symptoms of depression, and both animal and human research supports this prediction. For example, adult female rats exposed to uncontrollable stress showed deficits in hippocampus-dependent learning that were reversed following chronic treatment with fluoxetine (Leuner et al., 2004). Studies of depressed elderly patients also showed that fluoxetine effectively alleviated cognitive deficits while TCAs impaired or had no significant effect on cognition (Fairweather et al., 1993; Kerr et al., 1993). It is therefore plausible that the fluoxetine-induced increase in dorsal cell proliferation in OVX females relates to the positive effect of fluoxetine on cognition in depressed patients.

Long-term OVX altered cell proliferation in the ventral dentate gyrus

The current study found that long-term OVX did not have a significant effect on cell proliferation in the dorsal dentate gyrus but tended to increase proliferation in the ventral dentate gyrus of OVX/water controls. This is partially inconsistent with previous findings that, while short-term OVX (< 7 days) initially suppressed cell proliferation (Banasr et al., 2001; Tanapat et al., 1999), there was no significant effect on cell proliferation following long-term OVX (≥ 3 weeks; Banasr et al., 2001; Green and Galea, 2008; Tanapat et al., 2005; Lagace et al., 2007). However, none of the previous studies examined the dorsal and ventral dentate gyrus separately, which may have occluded possible regional differences in cell proliferation. When we examined cell proliferation in the overall dentate gyrus (combining the dorsal and ventral regions) we did not see a significant difference in cell proliferation between Sham/water and OVX/water controls ($p = 0.043$, data not shown; Bonferroni correction significant p-value is $p = 0.01$).

The increased cell proliferation in the ventral dentate gyrus of OVX/water controls may have been under adrenal hormone control. In rats, high adrenal hormone levels suppressed cell proliferation, while adrenalectomizing rats to eliminate adrenal hormones upregulated cell proliferation (Gould et al., 1992). In the current study, OVX/water compared to Sham/water controls showed significant adrenal hypotrophy, which is indicative of low levels of circulating adrenal hormones (Shinoda et al., 2002; Ramaley et al., 1976). It is therefore possible that lower levels of adrenal hormones in OVX/water controls led to upregulation of cell proliferation in this group of animals. Given the importance of the ventral hippocampus in stress and emotion (Bannerman et al., 2004), it is not surprising that the increase in proliferation was limited to the ventral dentate gyrus.

In the current study, OVX animals treated with imipramine and fluoxetine did not show an antidepressant-induced increase in ventral cell proliferation above OVX/water baseline levels. While it is possible that the lack of effect of antidepressants on cell proliferation in OVX animals was due to a ceiling effect, we do not think this is likely because previous research has shown that levels of cell proliferation in adult female rats can be more than five times greater than the levels observed in the current study (Jessberger et al., 2007).

Imipramine treatment increased immature neurons in the ventral dentate gyrus of OVX female rats

In the current study, treatment with imipramine significantly increased the number of immature neurons (DCX-labeled cells) in the ventral dentate gyrus of OVX animals. DCX-labeled cells are expressed in the 1-21 days preceding perfusion, with maximal DCX expression occurring at 4-7 days (Brown et al., 2003). In the current study this period of maximal DCX

expression corresponds to days 4-7 of antidepressant treatment. A seminal study by Malberg et al. (2000) found that antidepressants increased cell proliferation after chronic (≥ 14 days) but not acute or subchronic (1 or 5 days) exposure. Thus, the majority of DCX-labeled cells in this experiment would have been born during a time period of subchronic antidepressant administration where, based on Malberg et al. (2000), we would not expect to see an antidepressant-induced increase in cell proliferation or immature neurons. Consistent with the timeline of DCX expression, we did not see an increased number of immature neurons in the ventral dentate gyrus of Sham animals receiving imipramine, or the dorsal dentate gyrus of OVX animals receiving fluoxetine, despite seeing increased cell proliferation (Ki67 labeling) in these two groups after chronic (21 days) antidepressant treatment. Interestingly, we did see an increase in immature neurons in the ventral dentate gyrus of OVX animals receiving imipramine. This suggests that in OVX animals, a large number of immature neurons were produced during subchronic imipramine treatment.

Examination of DCX maturational stage data showed that all groups had significantly more DCX-labeled cells that expressed a relatively mature (compared to intermediate or immature) phenotype consisting of long processes with multiple branch points, which is consistent with previous research (Plumpe et al., 2007; Epp et al., 2011). However, OVX animals treated with imipramine showed greater maturity of DCX-labeled cells in the ventral dentate gyrus: they tended to have more neurons with long/branched processes compared to all other groups and had significantly more neurons with medium processes compared to OVX/water controls. The greater number of neurons expressing relatively mature phenotypes in the ventral dentate gyrus may indicate that imipramine increases the maturational rate of DCX-labeled immature neurons in OVX animals. The current results suggest that in OVX animals,

imipramine increases the number of immature neurons produced during subchronic treatment as well as the maturation rate of these neurons.

In the present study, we saw that imipramine treatment upregulated DCX-labeled immature neurons in OVX animals, but no significant increase in Ki67-labeled cells was seen. Because the timelines of Ki67 and DCX expression are very different, it is not surprising to see changes in one marker independent of changes in the other. The lack of proliferation (Ki67 labeling) in OVX animals after chronic imipramine treatment could be due to the presence of a compensatory mechanism that works to downregulate cell proliferation in response to an original increase in proliferation (Nowakowski and Hayes, 2008). In support of this hypothesis, research has shown that OVX females treated with exogenous estradiol benzoate showed an increase in cell proliferation 4 hours, but not 48 hours, after treatment (Ormerod et al., 2003). It is therefore possible that the absence of ovarian hormones in OVX females causes a dysregulation of normal neurogenic processes, such that treatment with imipramine induces an initial burst of immature neurons followed by subsequent suppression or normalization of cell proliferation levels. Notably, the increase in immature neurons in OVX animals receiving imipramine was seen only in the ventral dentate gyrus, again suggesting that the ventral dentate gyrus may be more sensitive to the antidepressant effects of imipramine than fluoxetine.

Chronic imipramine treatment increased cell proliferation and immature neurons in the ventral, but not dorsal, dentate gyrus

The current study found that chronic treatment with imipramine increased cell proliferation and immature neurons in the ventral, but not dorsal, dentate gyrus, which is consistent with various lines of research that implicate the ventral hippocampus in stress and

emotion (Bannerman et al., 2004). Lesion studies found that ventral, but not dorsal, hippocampus lesions produced anxiolytic effects on various behavioural tests (i.e. novelty suppressed feeding test: McHugh et al., 2004). There are also regional differences in anatomical connectivity of the hippocampus, with the ventral, but not dorsal, hippocampus sending projections to the prefrontal cortex, amygdala, and regions involved in HPA axis function (Fanselow and Dong, 2010). The ventral hippocampus also has a greater density of norepinephrine and serotonin innervation compared to the dorsal dentate gyrus (Gage and Thompson, 1980). Lastly, chronic antidepressant treatment upregulated cell proliferation to a greater extent in the ventral compared to the dorsal dentate gyrus (Banasr et al., 2006; Jayatissa et al., 2006). These data are consistent with the current finding that imipramine, which inhibits the reuptake of both norepinephrine and serotonin, upregulates cell proliferation in the ventral, but not dorsal, dentate gyrus.

Contrary to the ventral hippocampus, the dorsal hippocampus is primarily involved in learning and cognition. The dorsal hippocampus sends projections to areas of the brain (such as the dorsal region of the anterior cingulate cortex) involved in cognitive processes such as learning, memory, and navigation (Fanselow and Dong, 2010). Furthermore, lesion studies found that dorsal, but not ventral, hippocampus lesions interfered with spatial learning and memory (Morris water maze: Moser et al., 1993; McHugh et al., 2004). Taking into account the various lines of research outlining differences between the dorsal and ventral hippocampus, it is possible that the ability of antidepressants to upregulate neurogenesis in the ventral dentate gyrus may have more of an impact on the alleviation of depressive symptoms. Conversely, neurogenesis in the dorsal dentate gyrus may relate to the ability of antidepressants to alleviate cognitive symptoms of depression.

Compared to Sham females, OVX females had lower adrenal to body weight ratios that were restored following chronic imipramine and fluoxetine treatment

In the current study we calculated adrenal to body weight ratios to examine whether antidepressants affect the interplay between ovarian and adrenal hormones. We found that OVX/water controls had significantly smaller adrenal to body weight ratios than Sham/water controls, which is indicative of low levels of circulating adrenal hormones (Shinoda et al., 2002; Ramaley et al., 1976). This finding is consistent with previous animal research (Shinoda et al., 2002), as well as with human research that found that bilateral ovariectomy in premenopausal women suppressed HPA activity (De Leo et al., 1998). We also found that chronic treatment with both imipramine and fluoxetine reversed the OVX-induced adrenal hypotrophy and restored adrenal ratios to levels comparable to Sham/water controls. This is partially consistent with animal research that showed that chronic estradiol replacement reversed the OVX-induced decrease in adrenal to body weight ratios (Shinoda et al., 2002). It is also consistent with human research that showed that antidepressants normalized HPA axis dysfunction (Nickel et al., 2003; Ising et al., 2007). Taken together, our results suggest that ovarian hormones modulate the effect of antidepressants on adrenal hormones and HPA axis function. Future research examining serum corticosterone levels and dexamethasone suppression testing in Sham and OVX animals receiving antidepressants is warranted and may provide additional insight into ovarian hormone-mediated antidepressant regulation of HPA axis function.

Conclusion

The aim of the current experiment was to determine whether the presence or absence of ovarian hormones modulated the ability of two types of antidepressants to upregulate

hippocampal cell proliferation in the dorsal and ventral regions of the dentate gyrus. Here we show that chronic treatment with imipramine increases cell proliferation in the ventral dentate gyrus of females with intact ovarian hormone functioning. Conversely, ovariectomized females with very low levels of ovarian hormones show no proliferative response to imipramine in the ventral dentate gyrus, despite showing an increase in the total number of immature neurons in the ventral dentate gyrus. Contrary to treatment with imipramine, the effect of fluoxetine on cell proliferation is limited to the dorsal dentate gyrus of ovariectomized females. This study demonstrates that ovarian hormones modulate antidepressant-induced cell proliferation in a drug- and region-specific manner in females, and highlights the importance of considering sex and ovarian hormone status when examining the effect of antidepressants on hippocampal neurogenesis. Ovariectomy can also be used as a surgical model of human menopause, where low ovarian hormone levels seen following ovariectomy mimic the low ovarian hormone levels seen in post-menopausal women. Thus, the current findings may help elucidate a potential mechanism of action underlying antidepressant efficacy in women. Specifically, the current data suggest that normally cycling levels of ovarian hormones facilitate a proliferative response to imipramine, which might underlie behavioural symptom alleviation in premenopausal women with normal levels of ovarian hormones. Conversely, the lack of a significant effect on cell proliferation in ovariectomized females may be a potential mechanism of action underlying the decreased antidepressant efficacy in postmenopausal women with depression.

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