ANDROGENS AND NEUROPLASTICITY: CONTRIBUTIONS TO THE PATHOGENESIS AND TREATMENT OF DEPRESSION

by

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ABSTRACT

Depression is a devastating neuropsychiatric disease that has profound effects on neural structure and function, however the pathogenesis and modes of effective treatment remain poorly understood. Stress is the primary preceding factor in depression, leading to profound deficits in neurophysiology, particularly in the hippocampus. Depressed patients show reduced hippocampal neuroplasticity, while antidepressant treatment enhances both neurogenesis and the expression of proteins that mediate plasticity such as the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). Interestingly, men are half as likely as women to develop depression, where androgens appear to confer resiliency in males, as hypogonadal men are more likely to develop depression and supplementation of testosterone shows antidepressant efficacy. Little is known about the neurological underpinnings of this profound sex difference, however androgens influence the stress response and enhance hippocampal neurogenesis. The experiments in this thesis aimed to examine the role of androgens in the pathogenesis and treatment of depression using an animal model, with a specific eye toward the impact on hippocampal neurogenesis and neuroplasticity, and whether neuroplasticity mediated through PSA-NCAM is essential to antidepressant efficacy. In Chapter 2, surgically-induced hypogonadism potentiates the expression of depressive-like endophenotypes in male rodents within a chronic unpredictable stress (CUS) model of depression. Hypogonadal males showed potentiated behavioural, endocrine, and neurophysiological depressive-like phenotypes, including reductions in hippocampal neurogenesis and the expression of PSA-NCAM, compared to intact males. In Chapter 3, the hypogonadism-induced susceptibility to depressive-like phenotypes following CUS is largely inhibited by supplementation with testosterone. Testosterone treatment ameliorated physiological and endocrine phenotypes while showing

independent antidepressant-like effects and facilitating the efficacy of an antidepressant drug in some measures. In Chapter 4, the enzymatic cleavage of the polysialic acid moiety from NCAM completely inhibits the behavioural efficacy of antidepressant treatment, while also serving to attenuate the survival of newly generated hippocampal neurons. Collectively, this body of research demonstrates the protective effects of androgens against the development of depression in males, coinciding with enhanced hippocampal neuroplasticity, and delineates an essential role for neuroplasticity mediated through PSA-NCAM in antidepressant action.

PREFACE

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3α-diol	5α-androstane-3α, 17β-diol
3β-diol	5α-androstane-3β, 17β-diol
5-HT	5-hydroxytryptamine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AR	Androgen receptor
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA	Cornu Ammonis
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CUS	Chronic unpredictable stress
DAB	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DHT	Dihydrotestosterone
E_2	Estradiol
E. coli	*Escherichia coli*
EC	Entorhinal cortex
EndoN	Endoneuraminidase N

ER	Estrogen receptor
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
FST	Forced swim test
GABA	γ-Aminobutyric acid
GCL	Granule cell layer
GDX	Gonadectomy
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
HAM-D	Hamilton Depression Rating Scale
HRT	Hormone replacement therapy
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
I.P.	Intraperitoneal
ICV	Intracerebroventricular
IEG	Immediate early gene
LTD	Long term depression
LTP	Long term potentiation
LH	Luteinizing hormone
MAM	Methylazoxymethanol
MAOI	Monoamine oxidase inhibitor
MPOA	Medial preoptic area
MR	Mineralocorticoid receptor

mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCAM	Neural cell adhesion molecule
NDS	Normal donkey serum
NeuN	Neuronal nuclei
NHS	Normal horse serum
NIH	National institute of health
NMDA	N-methyl-D-aspartic acid
NNT	5-Nonyloxytryptamine oxalate
NSF	Novelty-suppressed feeding
OD	Optical density
OFT	Open field test
NRS	Normal rabbit serum
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PSA	Polysialic acid
PSD-95	Postsynaptic density protein 95
PSA-NCAM	Polysialylated neural cell adhesion molecule
PVA-DABCO	Polyvinyl alcohol-1,4-Diazabicyclo[2.2.2]octane
PVN	Paraventricular nucleus
RCT	Randomized control trial
ROI	Region of interest

S.C.	Subcutaneous
SD	Sprague-Dawley
SEM	Standard error of the mean
SGZ	Subgranular zone
SNRI	Serotonin–norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SVZ	Subventricular zone
TBS	Tris buffered saline
TCA	Tricyclic antidepressant

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1. GENERAL INTRODUCTION

1.1 Major Depression

Depression is a devastating neuropsychiatric disease that is both prevalent - with a lifetime incidence of 20% (Ustun et al., 2004) - and costly, accruing an estimated $14 billion in annual costs to Canadians (Akhtar-Danesh and Landeen, 2007). Depression is often a recurrent, life-long condition, with greater than 75% of patients with major depression remitting and showing exacerbation of depressive symptoms over time (Frank et al., 1990). Indeed, depression is now the second leading cause of disability in the world (Ferrari et al., 2013), and in the absence of successful treatment 15% of depressed individuals succumb to suicide (Guze and Robins, 1970).

Unipolar depression is a heterogeneous, spectrum disorder, that includes two primary and distinct depressive syndromes in the DSM-V, based on the pattern of psychologic and neurovegetative symptoms; melancholic and atypical (American Psychiatric Association., 2013). Pure melancholic depression affects approximately 30% of depressed patients and consists of symptoms such as intense anxiety, anhedonia, weight loss, feelings of worthlessness and helplessness, rumination, and negative outlook on the future (Levitan et al., 1997). Whereas atypical depression affects approximately 15-30% of depressed patients, and includes symptoms of emotional reactivity, weight gain, and hypersomnia (Levitan et al., 1997). The subclasses also respond differently to antidepressant treatment; atypical depression shows better response rates to monoamine oxidase inhibitors (MAOIs) and selective serotonin reuptake inhibitors (SSRIs), than to tricyclic antidepressants (TCAs) (Stewart, 2007). On the other hand, studies have shown that melancholic depression shows better response rates to TCAs compared to treatment with SSRIs (Roose et al., 1994; Perry, 1996; Anderson, 2000). Interestingly, atypical depression is 2-

3x more common in women, while melancholic depression is significantly more prevalent in men (American Psychiatric Association., 2000; Hildebrandt et al., 2003).

Although depression is highly prevalent and carries a large burden of disease, the pathoetiology of depression is poorly understood due to the heterogeneity in symptomology and treatment efficacy. Moreover, existing antidepressants are not ideal, as only a segment of patients are effectively treated and the therapeutic onset is delayed (Byrne and Rothschild, 1998; Pineyro and Blier, 1999; Trivedi et al., 2006). The exact mechanism of antidepressant drugs also remains to be elucidated; while there are several theories, including modulation of monoaminergic neurotransmission and the upregulation of adult hippocampal neurogenesis, no theory adequately explains how exactly antidepressant drugs alleviate mood disorders (Berton and Nestler, 2006). Thus, the development of novel, more efficacious antidepressant drugs requires a better understanding of the pathophysiological basis of depression and mechanisms of current antidepressant drugs. The great financial, societal, and human cost of depression warrants great scientific effort to elucidate the factors underlying the pathogenesis of depression and to development of more efficacious treatments.

In the next sections I will expand on the current understanding the pathogenesis of depression, assessing the effects of stress and the hormonal milieu on the susceptibility of depression. Further, I will outline the current understanding of antidepressant action with special attention toward the contributions of monoaminergic neurotransmission and neurogenesis, culminating in the neuroplasticity theory of depression which aims to bridge the prevailing theories and provide novel insight toward the pathogenesis, as well as the treatment of depression. In the experiments outlined in this thesis I aim to explore the contribution of

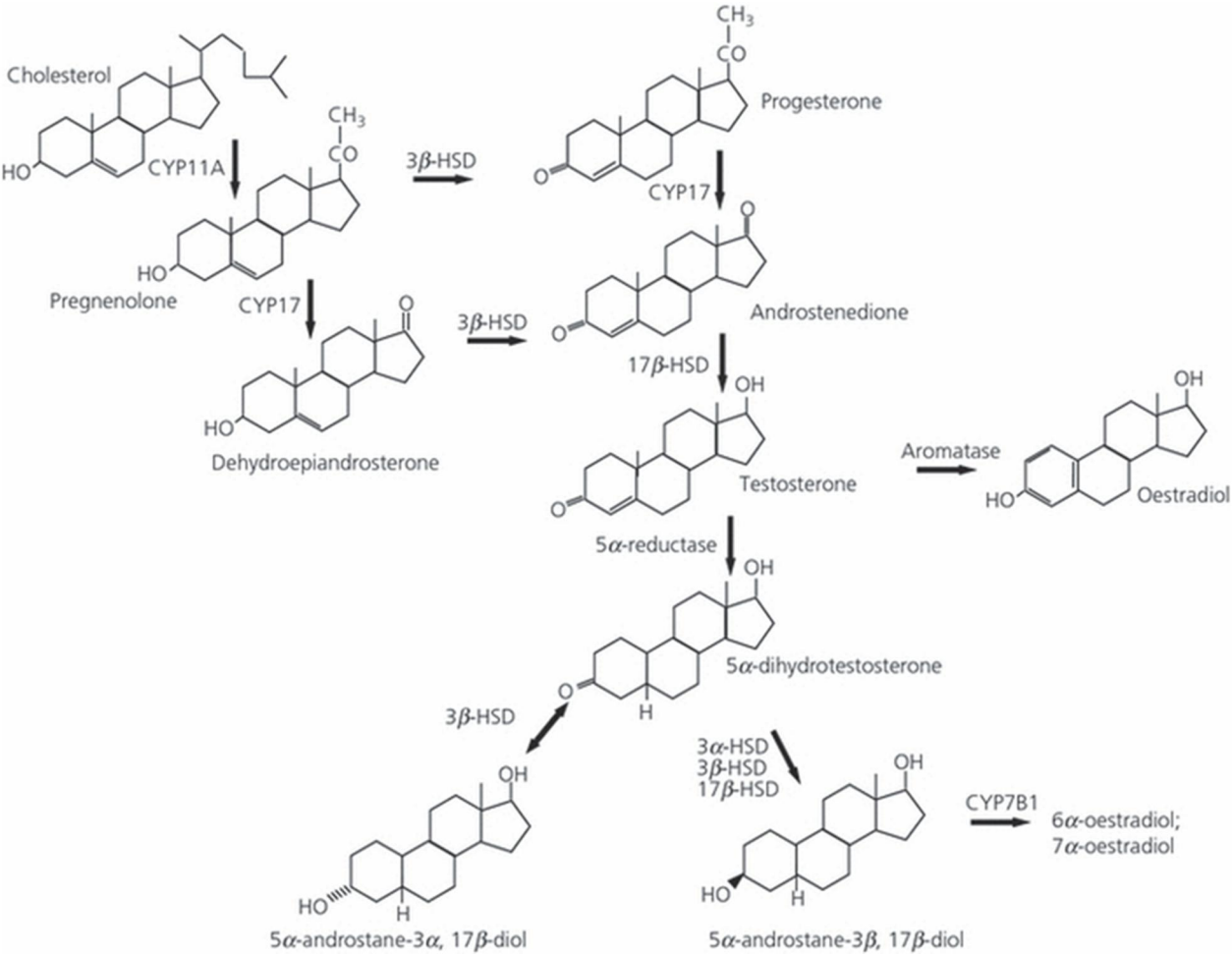
androgens and neuroplasticity to the pathoethiology of depression (Chapter 2) and to antidepressant efficacy (Chapters 3 and 4).

1.2 Sex Differences in Major Depression

Women are twice as likely as men to develop major depression (Weissman et al., 1993; Gutierrez-Lobos et al., 2002). Sex differences also exist in both the symptomology of depression and prevalence of comorbid disorders (Rodgers et al., 2014). Given such a marked difference between the sexes there must be factors either underlying susceptibility in females or conferring resiliency in males. The pronounced sex difference in the prevalence of depression is undoubtedly influenced by psychosocial factors in both women and men. For example, women encounter unique social burdens and face higher rates of physical and sexual abuse that are associated with increased rates of depression (Nolen-Hoeksema, 1990; Krakow et al., 2000). Men, on the other hand, are less likely to report depression than women, and veil their depression with drug or alcohol abuse (Orengo et al., 2004). However, there is also strong evidence indicating biological differences may increase the susceptibility of women and/or confer resiliency in men to the development of depression (Nolen-Hoeksema, 1987).

A primary focus of research regarding the neurobiological basis of sex differences in depression are gonadal hormones. The levels of estrogens in women, and androgens in men, are both associated with the pathoetiology of depression (Vogel et al., 1978; Galea et al., 2013). While evidence exists to support both psychosocial and biological influences, this thesis will explore the theory that suggests men may be less susceptible to the deleterious effects of stress, and thus by extension depression, due to the effects of androgens (Young, 1998).

Figure 1.1: Diagram of the synthetic pathway for androgens. Reprinted with permission form (Handa et al., 2009).



Androgens are the predominant gonadal hormones in men, derived from cholesterol via progestins, they include among others testosterone, androstenedione, and the testosterone metabolite produced via 5α-reductase, 5α-dihydrotestosterone (DHT). Testosterone and androstenedione may also be converted, via aromatase, to 17β-estradiol. 17β-estradiol is the most

biologically active of the estrogens in men and women (Nelson, 2011). Androgens are produced primarily in the Leydig cells of the testes, and transported through the circulatory system via sex hormone-binding globulin produced in the liver (Waterman and Keeney, 1992). Androgens act via the androgen receptor (AR), which is classically expressed in the cytoplasm then, when bound by either testosterone or the higher-affinity DHT, translocates to the nucleus and functions as a transcription factor to directly regulate gene expression via AR binding domains (Heinlein and Chang, 2002; Bennett et al., 2010). ARs also have a non-genomic pathway to induce rapid signalling following the binding of a ligand (Heinlein and Chang, 2002). Though DHT does not bind or activate estrogen receptors (ERs), it can be further metabolised by cytochrome P450 enzymes to 5α -androstane-3α, 17β-diol (3α- Diol) or 5α- androstane-3β, 17β-diol (3β- Diol), both of which show affinity for ERs (Handa et al., 2008) (see Figure 1.1).

There is evidence showing that androgen levels in men are associated with depression. For example, there is an increased incidence of depression in males coinciding with the age related decline in testosterone levels (Shores et al., 2004; Shores et al., 2005; McIntyre et al., 2006; Shores et al., 2009). Similarly, young and middle-aged men in a hypogonadal state are far more susceptible to developing depression (Veras and Nardi, 2010; Westley et al., 2015), portending protective effects of testosterone against the development of depression. Testosterone has also shown antidepressant effects, as testosterone replacement therapies have some efficacy in alleviating depressive symptoms in hypogonadal men (Seidman and Rabkin, 1998; Shores et al., 2009; Zarrouf et al., 2009). A meta-analysis in men indicated that testosterone therapy improved overall depression scores (Hamilton depression rating scale; HAM-D) after examining 7 studies that fulfilled their criteria (Odds ratio 0.40). Testosterone therapy showed improvement in depression scores in hypogonadal men, men with HIV/AIDS, and a mild improvement in

eugonadal men (Zarrouf et al., 2009). Similarly, a meta-analysis of post-menopausal women shows testosterone treatment improves mood scores; both alone, and in combination with estrogens (Zweifel and O'Brien, 1997). Testosterone replacement has also shown efficacy as an adjunct treatment to clinical antidepressants in cases of treatment-resistant depression (Seidman and Rabkin, 1998; Pope et al., 2003).

However, androgen therapies are not always seen to be effective for men suffering depression (Seidman et al., 2001a; Pope et al., 2010). Studies differ widely on the administration of testosterone (topical gel appears more efficacious than I.M. injection), time course, age of participants, depression scales, and what is considered an effective response rate (the meta-analysis used 50% effective change in HAM-D), which may all contribute to findings of the efficacy of testosterone treatment (Zarrouf et al., 2009). Furthermore, individual differences, such as the glutamine (CAG) polymorphism of the AR, also appear to be related to the effects of testosterone on depression incidence (Seidman et al., 2001b; Harkonen et al., 2003). For example, testosterone levels correlate negatively with depressed mood in men with short CAG repeat length but not in men with longer CAG repeats (Seidman et al., 2001b). However, greater CAG repeat length is positively correlated with depressed mood in aged men with no significant difference in serum testosterone levels (Harkonen et al., 2003). Thus, while there is controversy over the antidepressant-like action of testosterone supplementation in depressed patients, the majority of randomized control trials show some effectiveness of testosterone supplementation that may be limited to subpopulations and subtypes of depression (Rodgers et al., 2014). This thesis partially explores the role of androgens in the vulnerability to the development of depressive-like phenotypes (Chapter 2) and whether supplementation with testosterone, alone or

in combination with an antidepressant drug, produces antidepressant-like effects in an agonadal and stress-based model of depression (Chapter 3).

1.3 Monoamine Theory of Depression

Much of the current understanding of depression has been built upon the serendipitous discovery of the mood-elevating effects of iproniazid, a monoamine oxidase inhibitor (MAOI), during clinical trials for anti-tuberculosis agents in the early 1950's (Selikoff et al., 1952; Crane, 1957). Around the same time the first tricyclic antidepressant (TCA), imipramine, a drug also known to modulate monoaminergic neurotransmission but originally intended to treat psychosis, was discovered to relieve depressive symptoms (Kuhn, 1958). Both compounds functioned to enhance monoaminergic neurotransmitters (primarily serotonin and norepinephrine) in the synapse, and therefore acted as a springboard for the rational design of drugs which specifically enhance the transmission of these neurotransmitters. These findings resulted in the development of the monoamine hypothesis of depression, which postulates that the pathophysiological basis of depression is due to the deficient activity of monoamines in the central nervous system (Hirschfeld, 2000; Lopez-Munoz et al., 2007; Fangmann et al., 2008). Further supporting the supposition that deficiency of monoamines underlies the pathogenesis of depression, where studies assessing agents that deplete synaptic levels of monoamines, such as the antihypertensive drug reserpine, precipitated depression in some patients (Muller et al., 1955). Conversely, depressed and suicidal patients show lower levels of monoamine metabolites than healthy individuals (Traskman et al., 1981; Asberg et al., 1984), an effect that disappears if depressed patients recover (Traskman-Bendz et al., 1984; Nikisch and Mathe, 2008).

While first generation antidepressant drugs can indeed be effective, remission rates still typically remain below 60% and treatment is associated with potentially severe side effects, causing a third of patients to discontinue treatment (Thase et al., 1995; Thase, 2003). Thus new drugs, such as selective serotonin reuptake inhibitors (SSRIs), have been developed in an attempt to enhance efficacy and reduce the side effects observed with the broader-acting first generation compounds. However, the ability of these newer drugs to alleviate depression is typically lower than that of MAOIs and TCAs (Thase, 2003). SSRIs are the primary first line treatment for patients with major depressive disorder (MDD), yet only a third of patients will respond to these drugs following initial treatment (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 (Byrne and Rothschild, 1998).

While it is known that antidepressants modify monoaminergic neurotransmission, it is not known how antidepressants exert their therapeutic effects, as the influence on monoamines occurs within hours of treatment but alleviation of depressive symptoms requires weeks of exposure (Quitkin et al., 1984; Quitkin et al., 1987; Anderson et al., 2000; Blier, 2003). The monoamine theory explains this delayed efficacy of treatment as the time required for the 5-HT_{1A} serotonin autoreceptor to sensitize and normalize serotonergic tone at the synapse (Blier et al., 1987; Stahl, 1994; Pineyro and Blier, 1999). However, disrupting, or ablating portions of the serotonergic system, fails to induce a depressive phenotype in animal models (Lieben et al., 2006), suggesting that the dysregulation of serotonergic neurotransmission is not the only underlying factor in depression.

Interestingly, sex differences are seen in antidepressant efficacy, as men have a better response to TCAs, while women have a better response to SSRIs during the reproductive years

(Kornstein et al., 2000; Martenyi et al., 2001). Although, these findings remain somewhat controversial, they suggest that sex, and sex hormones influence the etiology of depression, and subsequently the optimal mode of antidepressant action. Clinical research shows that a transient reduction in serotonergic neurotransmission triggers significantly greater depressive symptoms in women over men (Dluzen et al., 2008). On the other hand, depletion of noradrenergic and dopaminergic neurotransmission may induce greater depressive symptoms in males, although these findings are inconsistent and may be dependent upon medical and family history (Grevet et al., 2002; Ruhe et al., 2007).

Testosterone may also act to influence the transmission of monoaminergic neurotransmitters, including serotonin, noradrenaline, and dopamine (Ebinger et al., 2009). Conversely, testosterone supplementation may be effective in enhancing the efficacy of monoaminergic antidepressants, a hypothesis explored in Chapter 3. Given the prevalence of treatment-resistant forms of depression and the limited long-term efficacy of current drugs, a better understanding of the pathoetiology of depression and the mechanism of current antidepressant treatments is needed to facilitate the development of more efficacious pharmacological treatments. The pathoetiology of depression is explored in Chapter 2, while mechanisms of antidepressant efficacy are explored in Chapters 3 and 4.

1.4 The Neurobiology of Stress

Stress is generally defined as any real, or perceived, challenge to homeostasis (Chrousos, 2009). The challenges to homeostasis are presented by stressors, which are any external or internal adverse forces, and may be physical or emotional in nature (Chrousos, 2009). The physiological alterations resultant of exposure to stress facilitate the escape from, or removal of, the stressor.

Upon the presentation of a stressor the body rapidly activates release of epinephrine and norepinephrine from the adrenal medulla via the sympathetic nervous system serving to enhance arousal and mental acuity (Cahill et al., 1994). Furthermore, a state of insulin resistance is induced to provide glucose to the brain, which serves to improve cognitive faculties and enhance memory (McEwen and Sapolsky, 1995). This rapid activation of catecholamines, via neurons in the hypothalamus and brainstem, mediates the immediate fight-or-flight response to stress (Jansen et al., 1995).

Subsequent to the rapid sympathetic response, a more "delayed" neuroendocrine response, mediated by the hypothalamic-pituitary-adrenal (HPA) axis, is also initiated. Catecholiminergic projections from the brainstem to the hypophysiotrophic zone of the paraventricular nucleus (PVN) of the hypothalamus participate in the activation of the HPA axis (Plotsky et al., 1989). Hypophysiotropic neurons localized in the medial parvocellular subdivision of the PVN synthesize and secrete corticotropin-releasing hormone (CRH) and argenine vasopressin (AVP), which are in turn released from the median eminence into the hypopheseal portal system. AVP is transported to the posterior pituitary where it facilitates HPA activation and autonomic responses in the brain stem. CRH is transported in to the anterior pituitary where it binds the $CRHR_1$ receptor and induces the release of adrenocorticotropic hormone (ACTH) from corticotropes into the systemic circulatory system. The principle target for ACTH is the adrenal cortex, wherein binding of ACTH to the adrenocorticotropin receptor stimulates the synthesis and secretion of glucocorticoids (Smith and Vale, 2006). Two primary glucocorticoids are released via the HPA axis; cortisol and corticosterone. In humans and other primates the primary glucocorticoid is cortisol (Underwood and Williams, 1972), while rats and mice primarily produce corticosterone (Nelson, 2011).

Glucocorticoids serve to aid survival in the face of stress; mobilizing glucose stores, increasing cardiovascular tone, reducing energy output to growth and reproductive processes (including the production of gonadal hormones), reducing inflammation, and potentiating immune function while preventing immune system activity from reaching the level of autoimmunity (Munck and Naray-Fejes-Toth, 1994; Sapolsky et al., 2000). However, the catabolic processes initiated by the release of glucocorticoids necessitate the restriction of secretion to times of overt need (Herman et al., 2005). Negative feedback mechanisms are therefore in place to limit the duration and magnitude of glucocorticoid release. There are two known glucocorticoid receptors in the brain: the low-affinity glucocorticoid receptor (GR), and the high-affinity mineralocorticoid receptor (MR). The GR is highly expressed in multiple brain regions including the PVN, anterior pituitary, and corticolimbic structures including the amygdala, prefrontal cortex (PFC), and hippocampus (Ulrich-Lai and Herman, 2009). The GR is extensively bound during times of high glucocorticoid output, such as periods of stress, and is thought to play a key role in re-establishing basal HPA tone (Reul and de Kloet, 1985). The MR is expressed predominantly in the hippocampus and is extensively bound during times of basal HPA activity (Reul et al., 2000). A delayed genomic feedback mechanism and a rapid non-genomic rapid feedback mechanism both serve to regulate HPA activity following stress. The genomic mechanism is mediated through the activation of GRs in HPA-associated brain regions, including the hippocampus. Indeed, the expression of hippocampal GRs is essential for effective regulation of HPA tone (Anacker et al., 2011a). The feedback regulation of the HPA axis is essential to return the body to homeostasis and basal physiological function. Any disruption resulting in the dysregulation of HPA tone, may facilitate the development of disease states, including mood disorders, such as major depressive disorder.

1.4.1 Stress and major depression

Stress is cited as the leading cause of depression by depressed patients (Bale, 2006; Miller et al., 2007; Schule, 2007; Beck, 2008). Unipolar depression is associated with abnormal hypothalamic-pituitary adrenal (HPA) axis function such as hypersecretion, and abnormal diurnal secretion, of cortisol resulting in a flattened circadian rhythm, particularly in melancholic depression (Parker et al., 2003; Schule, 2007). Indeed, studies have shown the hypersecretion of CRH in depressed patients, particularly in the morning hours when glucocorticoid levels are at their nadir (Wong et al., 2000). Similarly, the negative feedback regulation of the HPA axis is disrupted in depressed patients, where the administration of CRH or the synthetic glucocorticoid dexamethasone fails to suppress HPA activity and therefore glucocorticoid release (Holsboer, 2000). In fact, dysregulation of the HPA axis is the most prominent endocrine change seen with depression and normalizing HPA function is one of the major targets of pharmacological therapies (Keck, 2006; Schule, 2007).

In patients with dysregulated HPA function successful remission is associated with the normalization of CRH and glucocorticoid output (Holsboer, 2000). In fact, stabilization of the HPA axis by antidepressants is associated with improved mood scores that either precedes or is coincident with the behavioural alleviation of depressive symptoms (Ising et al., 2007). Chronic antidepressant treatment actually serves to upregulate the expression of GRs in the hippocampus and hypothalamus, serving to decrease basal and stress-induced glucocorticoid secretion (Pariante and Miller, 2001). Interestingly, treatment with tricyclic antidepressants appears to improve HPA negative feedback to a greater extent than MAOIs or SSRIs (Pariante and Miller, 2001). There is also a sex difference in HPA dysregulation and remission from depression, with women showing better ties to remission from depression and HPA negative feedback correction

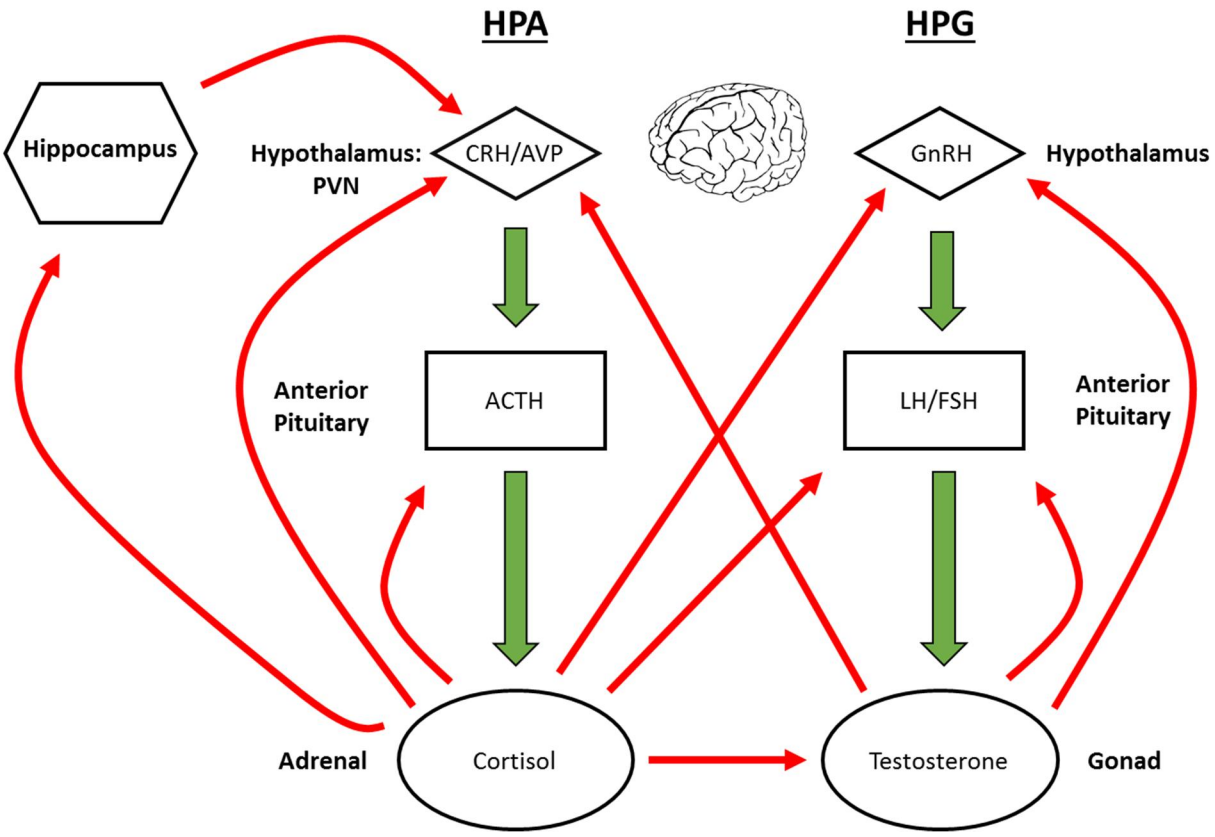
than men (Binder et al., 2009). These findings indicate that the hippocampus may play integral role in both the pathogenesis and successful treatment of depression.

1.4.2 Interaction between the HPA and HPG axes

The HPG axis functions in the production and regulation gonadal hormones in both sexes, wherein gonadotropin-releasing hormone (GnRH) is produced by GnRH neurons the hypothalamus and secreted at the median eminence into the hypophyseal portal system and transmitted to the anterior pituitary. Gonadotrophic cells in the anterior pituitary synthesize and secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the systemic circulatory system. Within the gonads LH and FSH function to facilitate the synthesis of androgens and estrogens (Vadakkadath Meethal and Atwood, 2005). Androgens and estrogens produced in the gonads function in the feedback regulation of the HPG axis, but importantly, they also have a functional interplay with the HPA axis (Viau, 2002).

The HPA axis generally functions on the HPG axis in an inhibitory manner in males. In both humans and rats, high levels of glucocorticoids suppress all aspects of HPG function, including decreasing GnRH secretion, reducing circulating levels of LH, and disrupting gonadal hormone production in males and females (Rivier and Rivest, 1991). There are also several mechanisms upstream that function in stress-induced HPG inhibition. In addition to multiple avenues of central inhibitory signaling that suppresses the synthesis of GnRH in the hypothalamus, CRH also acts to inhibit the production of GnRH, and decreases circulating levels of LH in rodents (Tsigos and Chrousos, 2002). However, there are reciprocal interactions where gonadal hormones modulate the HPA activity in a sex-specific manner (see Figure 1.2).

Figure 1.2: Diagram of the functional interaction and feedback mechanisms of the HPA and HPG axes in males. Green arrows indicate stimulatory. Red arrows indicate inhibitory.



The neuroendocrine response of females to stress is generally greater than that of males. For example, female rats show a larger and more prolonged release of ACTH and corticosterone in response to an acute stressor than males (Burgess and Handa, 1992; Goel et al., 2014). Females also have higher basal levels of corticosterone throughout the circadian cycle, and higher peak levels during the active phase (Critchlow et al., 1963; Atkinson and Waddell, 1997). Interestingly, the sex difference in HPA response is reduced by gonadectomy; in the absence of

gonadal hormones male rats show increased ACTH and corticosterone release in response to stress, while gonadectomy reduces ACTH and corticosterone levels in females (Goel et al., 2014). Treatment with testosterone, or DHT, in GDX males, and estradiol in GDX females, re-establishes the sex difference (Handa et al., 1994a; Handa et al., 1994b). The reverse is also true, as GDX males treated with estradiol show enhanced HPA reactivity and increased corticosterone levels, while GDX females treated with testosterone show reduced HPA reactivity and decreased corticosterone levels in response to stress (Goel and Bale, 2008). The inhibitory effect of testosterone are also dose-dependent, as testosterone levels are negatively correlated with neuroendocrine responses to stress (Goel et al., 2014). Intact males with higher naturally occurring testosterone levels show reduced ACTH and corticosterone secretion in response to stress than males with lower levels (Viau et al., 2003; Viau et al., 2005; Solomon et al., 2012).

Human studies also show women may produce a greater endocrine response to stress compared to men, though far less consistently. Findings are subject to numerous factors such as the type of stressor, test methodology, age, and the health of subjects (Goel et al., 2014). The testosterone-mediated feedback inhibition of HPA activity also holds true in human studies, as testosterone treatment suppresses CRH-induced cortisol release (Rubinow et al., 2005), though research in this area is limited.

Testosterone functions in the feedback inhibition of the HPA axis primarily at the level of the hypothalamus. Serum testosterone levels are positively correlated with GR binding in the medial preoptic area (MPOA) of the hypothalamus (Viau and Meaney, 1996). There are also AR-mediated mechanisms within the hypothalamus where DHT treatment into the PVN returns post-stress ACTH and corticosterone levels to that of an intact male (Lund et al., 2004). There is ample evidence to suggest that gonadal hormones play a prominent role in mediating HPA

reactivity, where estrogens function to potentiate the neuroendocrine response to stress and androgens act to inhibit HPA output in both males and gonadectomised females (Goel et al., 2014). These findings lend credence to the hypothesis that sex hormones function in the susceptibility or resilience to stress, and subsequently depression.

1.5 Animal Models of Depression:

There are numerous animal models of depression, which use various means to produce the desired phenotypes in animals, such as olfactory bulbectomy, or challenges to monoaminergic neurotransmission. However, these models are typically used with a specific eye toward producing only predictive (assessing the response to, and efficacy of, antidepressant drugs) or face validity (the extent to which putative symptomology resembles that of depression) (Willner, 1984; Willner and Mitchell, 2002).

Animal models of depression that capitalize on the association between stress and depression, also often have an extent of construct validity (theoretical viability and potential etiological viability) (Willner, 2005). While it is not possible to completely replicate the pathogenesis and symptomology of any affective disorder in rodents, it is possible to model depressive-like endophenotypes, including alterations in physiology, endocrinology, neurological measures, and behaviour. There are multiple means through which stress can be applied, including the chronic administration of exogenous corticosterone, chronic administration of a single stressor such as restraint or social isolation, but arguably the best model uses the chronic unpredictable application of multiple, varying, stressors. The chronic unpredictable stress (CUS) model, also referred to as chronic mild, variable, or intermittent stress, involves rodents receiving sustained exposure to stressors for a period typically lasting between 10 days to 8 weeks (Hill et

al., 2012). The CUS model serves to replicate the chronic exposure to multiple life stressors, which are described by depressed patients as the primary preceding factor for their depression (Kessler, 1997). Accordingly, CUS shows good face, construct, and predictive validity for melancholic depression, as the depressive-like endophenotypes produced, such as anhedonia, behavioural despair, reductions in body weight, HPA hyperactivity, and reductions in neural plasticity, resemble those observed in depressed patients (Willner, 1984; Hill et al., 2012). Importantly, each of these measures are also reversed by the chronic administration of antidepressant drugs, and therefore a CUS model presents an ideal background for the testing of novel antidepressant compounds (Vollmayr et al., 2007).

Exposure to chronic stress typically serves to reduce measures of neuroplasticity in the hippocampus. For instance, chronic stress exposure reduces dendritic length and complexity (Watanabe et al., 1992; Galea et al., 1997), spine density (Magarinos and McEwen, 1995), the expression of proteins associated with synaptic plasticity (Bessa et al., 2009; Muller et al., 2011), and adult neurogenesis in the hippocampus (Czeh et al., 2002; Alonso et al., 2004; Bessa et al., 2009). However there is a major caveat, as sex differences exist in the influence of stress on neural plasticity. For instance, chronic restraint stress produces atrophy of the apical CA3 dendrites of males but only in the basal CA3 dendrites of females (Galea et al., 1997). The effects of stress on hippocampal neurogenesis are also altered by sex, as males show reduced cell proliferation and survival while females actually show enhanced cell proliferation in response to chronic foot shock stress (Westenbroek et al., 2004). Therefore it is important to acknowledge that stress may affect the sexes differently, as outlined in the proceeding section.

The CUS model of depression also serves to model the central tenet of the monoamine theory of depression, where monoaminergic neurotransmission is altered in CUS exposed

rodents, as clearly outlined in the review by Hill et al., (2012). Though not a clear, unified picture, multiple studies show disrupted neurotransmission throughout multiple regions of the brain. For instance, in the hippocampus serotonergic neurotransmission is generally reduced, including the down regulation of the 5-HT1_A receptor. Similarly, though less consistently, dopaminergic and noradrenergic neurotransmission is often reduced. It must be stated that results are highly variable, this is perhaps not surprising given the heterogeneous nature of CUS protocols, which includes variability in length, composition, and administration of stressors. Nonetheless, CUS can elicit alterations in monoaminergic neurotransmission that are consistent with theory (Hill et al., 2012).

Endocrine changes also result from exposure to CUS, including HPA axis hyperactivity and disrupted diurnal HPA rhythm, resulting in increased basal corticosterone levels (Christiansen et al., 2012). Accordingly, hypothalamic levels of CRH are increased following CUS exposure and a reduction in the expression of GR occurs in the hippocampus, an effect that is consistent with the enhanced secretion of corticosterone and impaired negative feedback (Hill et al., 2012). The extensive neurochemical, neuroendocrine, and neuromorphological depressive-like phenotypes provide a solid physiological model of depression that also extend into measurable behavioural phenotypes.

1.5.1 Behavioural measures and the mechanisms of antidepressant action:

Numerous measures of animal behaviour have been developed to assess putative depressive-like phenotypes and to have predictive validity for the assessment of antidepressant compounds. Tests typically serve to assess a specific aspect of anxiety- or depressive-like behaviour, while conversely providing insight into the potential mechanism of a pharmacotherapy (Duman, 2010).

The behavioural tests used to measure depressive- and anxiety-like behaviour in this thesis are briefly summarized below.

1.5.1.1 The open field test (OFT)

The open field test (OFT) serves as a measure of locomotor activity and can also serve to investigate anxiety-like behaviour in rodents. The rat is placed into a novel open field environment, and the ambulatory behaviour is measured. Increased time spent in the periphery displaying thigmotaxis is indicative of anxiogenic behaviour, while anxiolytic treatment will result in more time spend in the center part of the open field. Anxiolytic drugs, such as benzodiazepines, consistently show reductions in thigmotaxis and increase time spent in the center of the field (Prut and Belzung, 2003). Androgens have been previously shown to produce anxiolytic effects male rodents (Frye and Seliga, 2001; Aikey et al., 2002; Roohbakhsh et al., 2011).

1.5.1.2 The novelty-suppressed feeding test (NSF)

When rodents are placed in a new environment they display hyponeophagia, in which feeding behaviour is suppressed (Shephard and Broadhurst, 1982). The novelty-suppressed feeding (NSF) test serves assess this novelty-induced hypophagia as a measure of both anxiolytic and antidepressant efficacy (Britton and Britton, 1981; Santarelli et al., 2003; David et al., 2009). For testing the rat is food-deprived, then placed in a novel, brightly-lit, open arena with a piece of food in the middle. The rat faces the conflict of staying in the periphery, or moving into the center of the anxiogenic environment to consume the food. The primary measure of the test is the latency to begin eating the food. Acute anxiolytic drug treatment serves to reduce the latency to

feed, however chronic antidepressant treatment is required to reduce the latency to feed, whereas acute antidepressant treatment instead produces anxiogenic effects (Santarelli et al., 2003; Vicente and Zangrossi, 2012). It has been shown that the efficacy of chronic antidepressant treatment, including with SSRIs and TCAs, is dependent upon hippocampal neurogenesis, as the ablation of hippocampal neurogenesis inhibits the antidepressant-induced reductions in latency to feed (Santarelli et al., 2003; Bessa et al., 2009; David et al., 2009).

In male rats, long-term gonadectomy has been shown to induce hypophagia in a manner that is reversed by treatment with testosterone, and to a lesser extent DHT (Gentry and Wade, 1976). Testosterone treatment in gonadectomised male rats has also been shown to reduce the latency to feed in a version of the NSF test that used a highly palatable food, and multiple days of testing (Carrier and Kabbaj, 2012a).

1.5.1.3 The forced swim test (FST)

The forced swim test (FST) serves as a putative measure of behavioural despair developed by Porsolt et al., (1977b) to assess depressive-like phenotypes while producing good predictive validity for the efficacy of antidepressant compounds. Rodents are placed in a cylindrical container of water, from which they cannot escape, their behaviours are then scored including climbing/struggling behaviour, swimming behaviour, and immobility (Slattery and Cryan, 2012). Treatment with antidepressant drugs serves to decrease the time spent immobile, and therefore increase swimming and/or struggling behaviour. Importantly, the enhancement of swimming behaviour is associated with increased serotonergic neurotransmission, whereas an increase in diving behaviour is associated with increased noradrenergic neurotransmission (Detke et al., 1995). Therefore the general mechanism through which an antidepressant compound may be

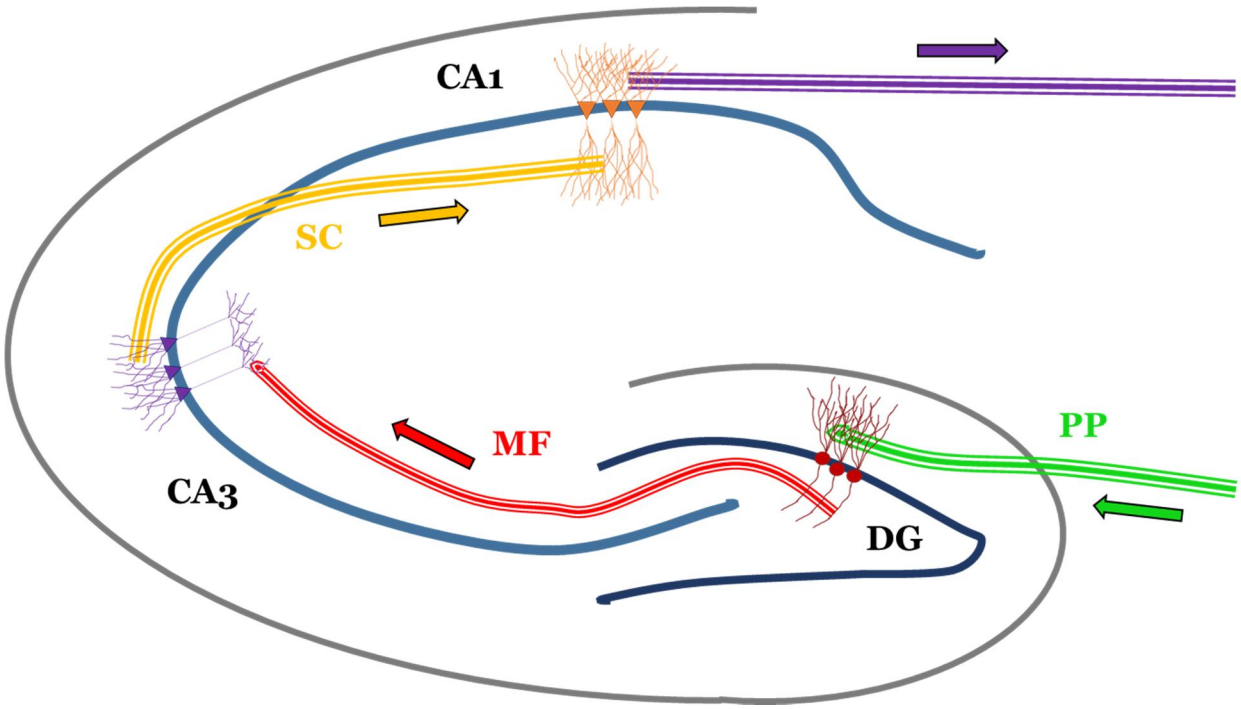
discerned from the FST. Immobility behaviour may also be modulated glucocorticoid levels, where enhanced corticosterone levels are associated with increased immobility behaviour, and disruption of hippocampal GR is associated with decreased immobility (Baez and Volosin, 1994; Korte et al., 1996). Androgen treatment serves to reduced immobility behaviour in both intact and GDX males, as well as female rats (Buddenberg et al., 2009; Frye and Walf, 2009; Carrier and Kabbaj, 2012a, b).

1.5.1.4 The sucrose preference test (SPT)

The sucrose preference test serves as a putative measure of anhedonia, wherein a depressive-like phenotype in rodents is indicated by a reduced preference for a sugar solution over water. The sucrose preference test is conducted as a two bottle choice in the home cage, one bottle containing a sucrose in water solution and the other containing just water, where the volume of each solution consumed over a set period of time is measured (Papp et al., 1991). The application of CUS reliably reduces sucrose preference, an effect that is reversed by antidepressant treatment (Willner et al., 1987; Muscat et al., 1992; Willner et al., 1992). The anhedonic effects elicited in the sucrose preference test are largely mediated by dopamine, as alterations in dopaminergic tone are observed in the forebrain following CUS exposure, treatment with dopamine antagonists reduces sucrose preference, and reductions in sucrose preference are reversed by dopamine agonists (Muscat and Willner, 1989; Papp et al., 1994; Willner et al., 1994). Androgens have also been shown to modulate sucrose preference in male rats, as gonadectomy reduces sucrose preference, while subsequent supplementation with androgens restores sucrose preference (Carrier and Kabbaj, 2012a, b).

Figure 1.3: Diagram of the hippocampal formation, including the trisynaptic circuit.

PP = Perforant pathway; MF = Mossy fiber pathway; SC = Schaffer collateral pathway



1.6 The Hippocampus

The hippocampus is a bilateral, subcortical structure located in the temporal lobes at the floor of the inferior horn of the lateral ventricles. The hippocampus has a laminar organization, consisting of two primary regions: the cornu ammonis (CA) containing four subdivisions; CA1, CA2, CA3, and CA4, and the dentate gyrus (DG), which interlock and fold into each other. The hippocampal formation also includes the entorhinal cortex and subiculum, which are connected to the CA and DG via the trisynaptic circuit. Axonal inputs enter the hippocampus from the

entorhinal cortex via the perforant pathway and form synapses on granule neurons of the dentate gyrus. Axonal projections from granule neurons are sent as mossy fibers to pyramidal neurons in CA3, which in turn project onto pyramidal neurons in CA1 via the Schaffer collateral pathway. Projections from CA1 are then sent to the subiculum, where projections then exit the hippocampal formation (see Figure 1.3). The dentate gyrus is composed of three layers: the granule cell layer, the molecular layer, and the hilus (or polymorphic layer). Along the border between the GCL and the hilus is a thin region of cells known as the subgranular zone (SGZ). Within the SGZ reside neural stem cells, which are capable of mitosis and the production of new neural cells in adulthood.

The function of the hippocampus is complex and varied. The hippocampus is involved in certain forms of learning and memory, such as spatial and working memory, with several forms being hippocampus-dependent (Shors et al., 2002). The hippocampus is also hypothesized to play a role in emotional processing, as the hippocampus is part of the limbic circuit, and disruption of normal hippocampal function is associated with emotional disturbances (Davidson et al., 2000). Interestingly, the hippocampus does not appear to act as a single, homogenous, structure, rather there appears to be a functional dissociation along the dorsoventral axis (Moser and Moser, 1998). Anatomical, gene expression, and behavioural studies point to a primary role in cognition for the dorsal hippocampus, while the ventral hippocampus is primarily involved in affective functions (Fanselow and Dong, 2010). Neuronal connectivity is also dissociated along the dorsoventral axis. The dorsal region shows a greater density of place cells involved in the coding of spatial location and reciprocal connections with regions prominently involved in cognitive processing and locomotion, while the ventral region shares connections with the amygdala, medial prefrontal cortex (mPFC), and hypothalamus in a manner primarily associated

with neuroendocrine regulation, stress, and affect (Herman and Cullinan, 1997; Fanselow and Dong, 2010).

1.6.1 Adult hippocampal neurogenesis

Altman first found neural stem cells in the brain of adult rodents more than fifty years ago (Altman, 1962), and neural stem cells have since been identified in a number of species, including humans (Eriksson et al., 1998). The production of new neurons in the adult brain is typically limited the subventricular zone (SVZ), which lines the lateral ventricles and sends newly generated cells along the rostral migratory stream to the olfactory bulb, and the subgranular zone of the hippocampus (Kaplan and Hinds, 1977). However, studies in human indicate that adult neurogenesis may not occur in the SVZ, and is limited to the hippocampus (Sanai et al., 2011; Spalding et al., 2013). Multipotent neural stem cells are capable of producing multiple types of both neurons and glia and are located in the SVZ (Reynolds and Weiss, 1992; Gage et al., 1995; McKay, 1997), while neural progenitor cells located in the SGZ divide more frequently, in a finite manner, and into a limited number of cell types, as they may only produce daughter cells of either a defined glial or neuronal lineage (Gage, 2000; van der Kooy and Weiss, 2000; Seaberg and van der Kooy, 2002, 2003).

Newly generated neurons may be identified through the administration of DNA-markers such as ^{3}H-thymidine, or a synthetic nucleoside such as 5-bromo-2-deoxyuridine (BrdU). Each marker is incorporated into the DNA of dividing cells during DNA synthesis, replacing thymidine nucleotides. The addition of BrdU into the DNA provides a unique epitope for antibody binding using immunocytochemistry. The controlled addition of the exogenous markers allows for a definitive timeline of neural development, as every cell undergoing DNA synthesis

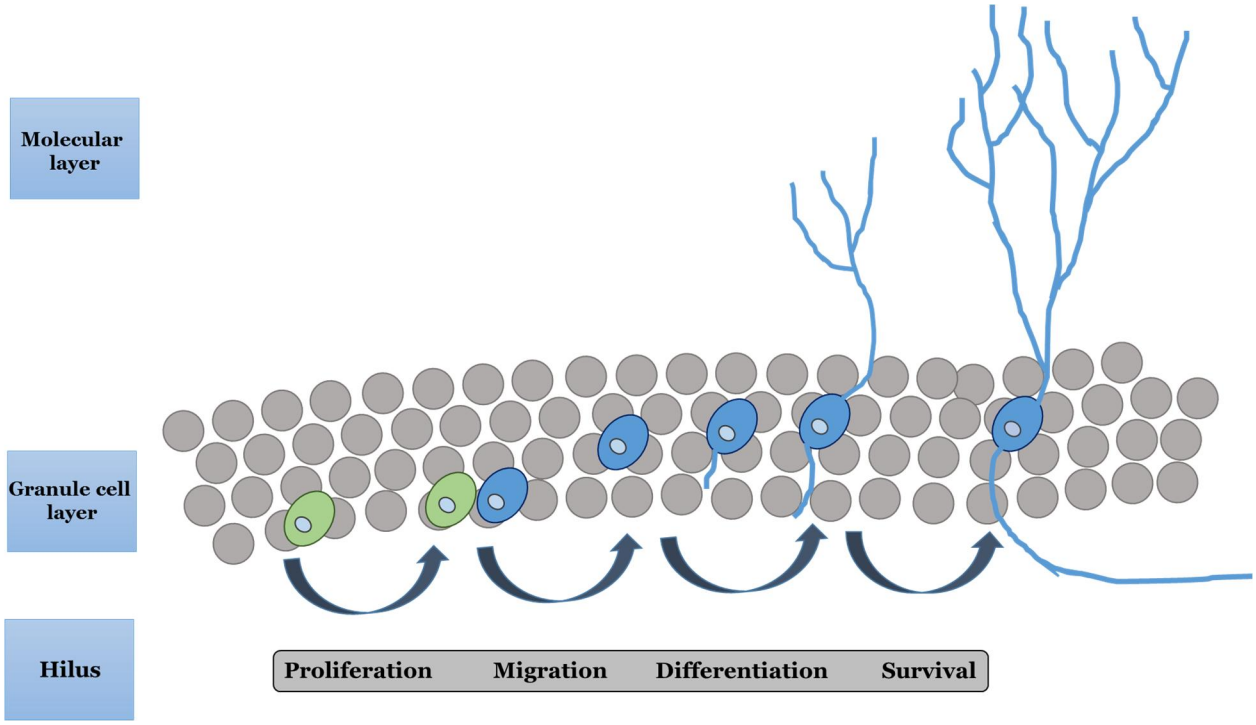
during the two hours after injection will be labelled. Labelled cells may be examined at time points ranging from hours, weeks, or even years later depending on the research question (Eriksson et al., 1998; Dayer et al., 2003; Kempermann et al., 2003).

There are also limitations in the use of exogenous markers as they become diluted as cells undergo mitosis and are only distinguishable from background for 4-5 divisions following administration, thereby limiting the population of cells that may be labelled (Stone et al., 1965; Prickaerts et al., 2004). Factors such as dose, toxicity, or the permeability of the blood-brain barrier may also disrupt the detection of true cell counts when using exogenous markers of DNA synthesis (Taupin, 2007). Exogenous markers must also be used in conjunction with endogenous markers, such as neuronal nuclei (NeuN) for mature neurons or glial fibrillary acidic protein (GFAP) for glial cells, in order to phenotype labelled cells. It is therefore essential that labelled cells be assessed with endogenous markers in order to demonstrate that neurogenesis has occurred.

The measurement of neurogenesis may also be achieved through the assessment of endogenous protein expression alone. Measurement of the Ki67 protein, which is expressed throughout the active phases of the cell cycle but absent in quiescent cells, may be used as a marker of cell proliferation (Zacchetti et al., 2003). The use of Ki67 in the measurement of cell proliferation also precludes issues with toxicity encountered with exogenous markers. Endogenous markers may also be used to identify and characterize both immature and mature neurons. Doublecortin (DCX) is a neuron-specific protein that functions in the stabilization microtubules in early mitotic neurons which may be used to measure immature neurons and assess neuronal morphology (Bechstedt et al., 2014). The NeuN protein is expressed by mature

neurons and functions in RNA splicing within the nucleus, and may be used in the characterization of mature neurons (Kim et al., 2009; von Bohlen und Halbach, 2011).

Figure 1.4: Diagram depicting the stages of adult neurogenesis in the dentate gyrus



It is important to note that neurogenesis, as defined here, requires the proliferation, migration, survival, and differentiation of newly generated cells into neurons (see Figure 1.4). Any number of internal and/or external factors may independently affect the proliferation of progenitor cells, migration, their differentiation into neurons, or their survival rates; including stress, gonadal hormones, or pharmaceuticals (Kempermann et al., 1998; van Praag et al., 1999; Malberg et al., 2000; Barker and Galea, 2008; Burgess et al., 2008; Pariante and Lightman, 2008; Anacker et al., 2011b; Surget et al., 2011; Wainwright et al., 2011). It is therefore essential to

understand which aspects of neurogenesis are being examined, and when they are being examined, in relation to experimental manipulation.

1.6.2 The hippocampus in the neurobiology of depression

The hippocampal formation is an area rich in mineralocorticoid (MR) and glucocorticoid receptors (GR) (McEwen, 1973). These receptors function in the maintenance of basal HPA tone, and in the regulation of negative feedback of glucocorticoid release during a stress response (Sapolsky et al., 1985a). Given this, it is not surprising that the hippocampus is particularly vulnerable to the effects of stress and depression (Sapolsky, 1986; Sapolsky et al., 1988). Imaging studies have shown depressed patients have reduced hippocampal volume which co-varies with the number of episodes and duration of the illness (Sheline et al., 1996; Sheline et al., 2003; McKinnon et al., 2009). Further, the meta-analysis by McKinnon et al., (2009) showed that only patients that had depression persist longer than two years, or had more than one episode of depression, showed significant reductions in hippocampal volume. However, reductions in volume were only found in children and middle-aged to older adults, where young adults showed no significant difference between depressed patients and control subjects. Interestingly, the times during life that the hippocampus is most vulnerable to the effects of chronic or recurrent depression, are also the time when gonadal hormones are lower.

Post-mortem studies of hippocampal tissue collected from depressed patients have shown alterations in gray matter density, reductions in neuropil, decreased neuron soma size, and decreased hippocampal neurogenesis (Stockmeier et al., 2004; Boldrini et al., 2009; Hercher et al., 2009; Boldrini et al., 2012a; Boldrini et al., 2013b; Cobb et al., 2013). These findings suggest that depression is associated with significant changes in both the number and connectivity of

hippocampal neurons, which may contribute to the reductions in hippocampal volume observed in imaging studies. However, the reduction in neurogenesis has been the primary focus of depression research with respect to the hippocampus, as investigation of the mechanisms of antidepressant efficacy beyond the monoamine theory point to significant role for newly generated hippocampal neurons, in both animal models of depression and human studies.

1.6.3 Adult hippocampal neurogenesis and depression

The neurogenic hypothesis of depression posits that impaired hippocampal neurogenesis precipitates the development of depression, and successful antidepressant treatment requires the enhancement of hippocampal neurogenesis (Jacobs et al., 2000). The neurogenic hypothesis of depression is predicated not only on the previous findings that chronic stress decreases hippocampal neurogenesis in animal models of depression (Gould et al., 1997; Santarelli et al., 2003; Bessa et al., 2009), but that antidepressant drugs can both prevent (Czeh et al., 2001; Alonso et al., 2004) and reverse (Malberg and Duman, 2003; Santarelli et al., 2003; Bessa et al., 2009) this effect. Interestingly, the neurogenesis-reducing effects of stress and neurogenesis enhancing effects of chronic antidepressant treatment are, though not exclusively, predominantly observed in the ventral region of the hippocampus (Tanti and Belzung, 2013).

The enhancement of adult hippocampal neurogenesis is dependent on chronic, but not acute, exposure to antidepressants, occurring in a manner that temporally coincides with the delayed clinical efficacy of these drugs (Malberg et al., 2000; Santarelli et al., 2003). This delayed enhancement of neurogenesis is mediated through the increased proliferation of neural progenitor cells, while the proportion cells that survive and differentiate into neurons remains constant (Malberg et al., 2000). Several classes of antidepressant drugs including SSRIs, TCAs,

and MAOIs increase neurogenesis in the hippocampus (Banasr et al., 2006; Dagyte et al., 2010). Moreover, a rodent model of the non-pharmacological antidepressant treatment of electroconvulsive therapy (ECT) also enhances neurogenesis in the adult hippocampus (Madsen et al., 2000; Hellsten et al., 2002). ECT is the most effective antidepressant treatment in depressed patients that are treatment resistant (Pagnin et al., 2004) and it can upregulate neurogenesis to levels nearly twice that of pharmacological antidepressants in animal models (Madsen et al., 2000).

Table 1.1: Selected publications showing the effect of stress/depression or antidepressant treatment on neurogenesis and the functional importance of neurogenesis in antidepressant efficacy.

(a) Animal models of depression

Reference	Species	Sex	Model	Antidepressant	Method of ablation	Summary of findings
Snyder et al. 2011 [96]	Mouse	M	Restraint	—	X-ray; transgenic	Neurogenesis-dependent regulation of HPA response to stress and behavioural measures (NSF, FST, and SC)
David et al. 2009 [64]	Mouse	M and F	Chronic corticosterone administration	Fluoxetine	X-ray	Neurogenesis-dependent (NSF) and -independent (OFT, FST) aspects of antidepressant efficacy
Bessa et al. 2009 [65]	Rat	M	Chronic mild stress	Imipramine; fluoxetine	MAM	Neurogenesis-dependent (NSF) and -independent (SC, FST) aspects of antidepressant efficacy. Significant alterations in neural plasticity associated with antidepressant efficacy
Surget et al. 2008 [92]	Mouse	M	Chronic unpredictable stress	Imipramine; fluoxetine		Neurogenesis-dependent (NSF, CS, ST) and -independent (A) aspects of antidepressant efficacy
Holick et al. 2008 [93]	Mouse	M	—	Fluoxetine	X-ray	Neurogenesis-independent effects of antidepressant efficacy
Airan et al. 2007 [94]	Rat	F	Chronic mild stress	Imipramine; fluoxetine	X-ray	Neurogenesis-dependent (NSF) and -independent (OFT) aspects of antidepressant efficacy
Alonso et al. 2004 [85]	Mouse	M	Chronic mild stress	Fluoxetine	—	Decreased cell proliferation in the DG, while chronic fluoxetine blocked this effect
Santarelli et al. 2003 [21]	Mouse	M	—	Imipramine; fluoxetine	X-ray	Neurogenesis-dependent (NSF) antidepressant efficacy
Czéh et al. 2002 [78]	Rat	M	Social subordination	—	—	Decreased cell proliferation and survival in the DG
Malberg et al. 2000 [9]	Rat	M	—	Fluoxetine; reboxetine; tranylcypromine; ECS	—	Chronic, but not acute, treatment with monoaminergic antidepressant and ECS increased cell proliferation in the DG

(b) Human studies of depression

Reference	Subjects	Sex	Population assessed	Antidepressant	Effect on neurogenesis
Cobb et al. 2013 [74]	Humans	M and F	Depressed patients postmortem	—	No significant difference in number of granule cells between depressed subjects and controls; decreased hippocampal volume correlating with duration of disease
Boldrini et al. 2013 [75]	Humans	M and F	Depressed patients postmortem	SSRIs; TCAs	Depression is associated with a decreased number of granule neurons, correlated with reduced DG volume. SSRI and TCA treatment increase granule neuron number and DG volume
Boldrini et al. 2012 [22]	Humans	M and F	Depressed patients postmortem	SSRIs; TCAs	Both antidepressant classes increase cell proliferation over untreated depressed patients and controls; NPCs associated with angiogenesis
Boldrini et al. 2009 [23]	Humans	M and F	Depressed patients postmortem	SSRIs; TCAs	Both antidepressant classes increase cell proliferation over untreated depressed patients and controls
Stockmeier et al. 2004 [76]	Humans	M and F	Depressed patients postmortem	—	Increased density of granule cells in the DG of depressed subjects compared to controls

NSF: novelty suppressed feeding; FST: forced swim test; SC: sucrose consumption; OFT: open field test; CS: coat state; ST: splash test; A: actimeter; MAM: methylazoxymethanol acetate (cytostatic agent).

Recent, post-mortem human studies have also shown antidepressant treatment enhances hippocampal neurogenesis (Boldrini et al., 2009; Boldrini et al., 2012b; Epp et al., 2013). Interestingly, one study shows treatment with TCAs increases cell proliferation levels beyond that of SSRIs (Boldrini et al., 2009), while another by the same research group shows a greater enhancement of proliferation by SSRIs over TCAs (Boldrini et al., 2012b). There is a similar lack of continuity in measurements of hippocampal volume following antidepressant treatment, where SSRIs have been shown to enhance hippocampal volume over TCA treatment (Boldrini et al., 2012b; Boldrini et al., 2013a), while a single study finds the reverse (Boldrini et al., 2009). It is therefore important to note that the majority of pre-clinical research has been conducted exclusively in male rodents, while post-mortem studies typically analyse both men and women together. One recent study has shown that antidepressant treatment only enhances the expression of DCX/NeuN ratio in women but not men, therefore demonstrating that pro-neurogenic effects of antidepressant treatment may not only depend on the mechanism of antidepressant action, but the sex of the person taking a particular antidepressant (Epp et al., 2013). The age of the research subjects is also important, as two studies have shown that antidepressants fail to enhance neurogenesis in older patients (Lucassen et al., 2010; Epp et al., 2013). Thus it is important to distinguish between findings in males versus females, and understand fundamental differences between the sexes in the pathogenesis and treatment of depression (see Table 1.1).

While it is accepted that chronic antidepressant treatment upregulates hippocampal neurogenesis, some studies have suggested that increased neurogenesis is absolutely necessary for antidepressant efficacy. Indeed, studies have shown that if hippocampal neurogenesis is reduced via localized irradiation antidepressants lose their efficacy in rodent models of depression (Santarelli et al., 2003). However there are caveats, as the use of the cytostatic agent

methylazoxymethanol (MAM) to reduce hippocampal neurogenesis failed to disrupt the behavioural efficacy of chronic antidepressant treatment on sucrose preference, or in the forced swim test, and antidepressant treatment still increased hippocampal volume as well as the expression of synaptic proteins (Bessa et al., 2009). Nonetheless, neurogenesis does seem to be required for the attenuation of anxiety-like behaviour as measured in the novelty supressed feeding (NSF) test; suggesting a role for neurogenesis in specific aspects of antidepressant efficacy (Santarelli et al., 2003). Indeed, further research has shown neurogenesis-dependent and –independent effects of antidepressant treatment, where hippocampal neurogenesis is only required for the alleviation of some anxiety/depressive-like behaviours (Airan et al., 2007; Holick et al., 2008; Surget et al., 2008; David et al., 2009). These findings point toward multifaceted and pleiotropic effects of antidepressants on neural circuitry. Interestingly anxiolytic drugs, such as benzodiazepines, also show efficacy in the NSF test with acute treatment; while antidepressants require chronic, neurogenesis-enhancing, treatment (Bodnoff et al., 1989). It is then possible that the reduction in glucocorticoids from anxiolytic treatment mediates the reduction in anxiety-like behaviour in the NSF test (Bizzi et al., 1984), and therefore antidepressant-induced neurogenesis may mediate a similar effect on glucocorticoid levels. Indeed this may be the case, as hippocampal neurogenesis buffers the stress response and normalizes glucocorticoid release after stress (Snyder et al., 2011). Snyder and colleagues found that inhibition of hippocampal neurogenesis, either transgenically or via irradiation, attenuated both the recovery of basal HPA tone following acute restraint stress and the normal suppression of glucocorticoid release during the dexamethasone suppression test (Snyder et al., 2011). Importantly, the ablation of neurogenesis does not alter the ability of the HPA axis to mount a response to stress (Santarelli et al., 2003). However, the ability of an antidepressant (fluoxetine)

to improve negative feedback and normalize HPA tone in a chronic unpredictable stress (CUS) model of depression is abolished by the ablation of hippocampal neurogenesis (Surget et al., 2011). Further, the transgenic enhancement of hippocampal neurogenesis is sufficient to ameliorate some measures of anxiety- and depressive-like behaviour in a chronic CORT administration model of depression (Hill et al., 2015). As such, there is likely a role for hippocampal neurogenesis in re-establishing normal HPA tone and regulating a normal HPA response to stressors; possibly through GR-mediated negative feedback. Thus the mechanisms underlying antidepressant efficacy may be partially elucidated through behavioural measures, and by the importance of neurogenesis within those measures.

1.6.4 Hippocampal neurogenesis and androgens

There is ample evidence showing that androgens influence neurogenesis in the adult hippocampus. In adult male rodents androgens reliably increase hippocampal neurogenesis via the modulation of cell survival (for review see Galea et al., 2013). Several studies have shown that the removal of androgens, via gonadectomy, causes a reduction in the number of newly generated neurons that survive to maturity in rodents (Spritzer and Galea, 2007; Spritzer et al., 2011; Wainwright et al., 2011; Hamson et al., 2013). On the other hand, supplementation with either testosterone or DHT ameliorates the effect of GDX on cell survival in males (Spritzer and Galea, 2007; Hamson et al., 2013), however some studies have shown no effect of testosterone supplementation on cell survival in GDX (Spritzer et al., 2011; Carrier and Kabbaj, 2012b) or intact males (Buwalda et al., 2010). The duration of androgen treatment appears to be critical in the promotion of cell survival. The studies that show enhancement of cell survival use 30 days of androgen administration (Spritzer and Galea, 2007; Spritzer et al., 2011; Wainwright et al., 2011;

Hamson et al., 2013), while those failing to enhance cell survival use 15 or 21-day courses of androgen treatment (Spritzer et al., 2011; Carrier and Kabbaj, 2012b). Given the length of exposure required to elicit an increase in cell survival, the effect is likely mediated through a genomic mechanism (Galea et al., 2013).

Androgens appear to have less of an influence on adult hippocampal cell proliferation, as several studies have shown that GDX fails to produce any significant change in cell proliferation levels, nor is there an effect of androgen supplementation (administration of testosterone or DHT) using either exogenous or endogenous markers of cell proliferation (Spritzer and Galea, 2007; Carrier and Kabbaj, 2012a; Hamson et al., 2013; Allen et al., 2015). Though not a significant effect, there is a trend toward testosterone supplementation reducing cell proliferation in intact male rats (Buwalda et al., 2010). Interestingly, another study has found that administration of finasteride, a 5α-reductase inhibitor that functions to inhibit the metabolism of testosterone to DHT, in intact male mice actually serves to reduce cell proliferation and the expression of DCX-immunoreactive neurons in the hippocampus (Romer et al., 2010). These findings demonstrate that androgen treatment has pro-neurogenic effects on cell survival in agonadal males, but potentially deleterious effects on cell proliferation in intact males. It is therefore possible that androgen levels that are either too low or too great, have detrimental effects on hippocampal neurogenesis in rodents.

A study in non-human primates has shown dissimilar results than those observed in rodents. Though there is no significant effect of gonadectomy on hippocampal cell proliferation, gonadectomised males showed increased cell survival and enhanced differentiation to a neural phenotype (Allen et al., 2014). However, it is important to note that the Rhesus Macaques used in that study were gonadectomised prior to puberty, and neurogenesis was not measured until

over two years after surgery. Given the pre-pubertal removal of gonadal hormones, and the long absence from androgens, any differences in hippocampal neurogenesis cannot be solely attributed to an AR-dependent neurogenic mechanism.

The androgenic regulation of cell survival within the dentate gyrus has been specifically attributed to the activation of the AR in rodents, as estrogenic metabolites of testosterone such as estradiol, produce no significant effect on cell survival (Spritzer and Galea, 2007; Carrier and Kabbaj, 2012b). Indeed, testosterone exerts an effect on neurogenesis via an AR-dependent mechanism, as treatment with the testosterone metabolite DHT, which has a higher affinity for AR than testosterone, produces an increase in neurogenesis. Moreover, the enhancement of neurogenesis by androgen treatment is blocked by the AR antagonist flutamide (Hamson et al., 2013). Similarly, testosterone treatment fails to enhance neurogenesis in rats containing a mutation of the AR that renders it non-functional (Hamson et al., 2013). It is important to note, that neither the administration of the AR-antagonist flutamide, nor the expression of the insensitive androgen receptor, altered levels of cell proliferation.

The modulation of androgens also alters the expression of neurotrophic factors and structural proteins associated with neuroplasticity in the hippocampus. Gonadectomised rats show reductions in genes associated with neurogenesis, neuroplasticity, and cell adhesion (Quintela et al., 2015). Conversely, testosterone supplementation in GDX male rats enhances the expression of cell adhesion molecules in the hippocampus (Monks et al., 2001). These findings indicate that androgens modulate hippocampal plasticity in the adult hippocampus beyond their influence on hippocampal neurogenesis. It is therefore pertinent to investigate the potential role of androgen-mediated hippocampal neuroplasticity in the pathogenesis and treatment of depressive-like phenotypes in an animal model of depression.

1.7 Cell Adhesion Molecules

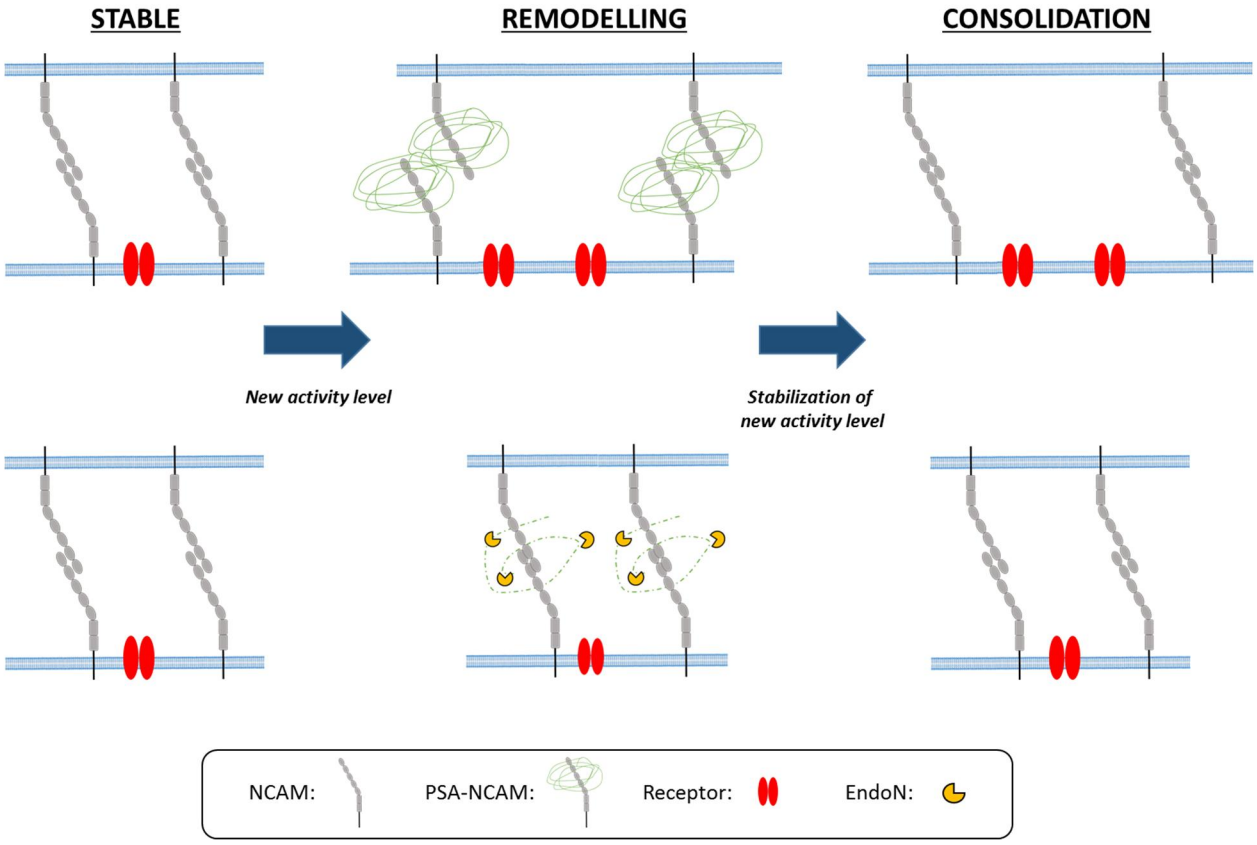
Neural plasticity is a complex and varied process; from neural development, the migration of newly generated neurons, dendritic modifications and synaptic modulation, many proteins facilitate and contribute to the malleability of neural tissue. Cell adhesion molecules (CAMs) are specialized proteins – typically expressed at the cell surface – which are important in synaptic function, synaptic plasticity, remodelling of neural circuits, and neurogenesis (Dalva et al., 2007). The structure, function, and expression of CAMs varies widely within the body and the nervous system. CAMs are categorized into two main groups, calcium-dependent and calcium-independent CAMs (Brackenbury et al., 1981). Calcium-dependent CAMs include integrins, cadherins, and selectins among others (O'Connell et al., 1996; Sjaastad and Nelson, 1997; van Roy and Berx, 2008). Calcium-independent CAMS are predominantly members of the immuglobulin superfamily and therefore share a protein structure similar to that of antibodies (Sandi, 2004). The categorization of the structure and function of each CAM protein involved in neural plasticity is beyond the scope of this thesis. Focus will be placed on the neural cell adhesion molecule (NCAM), given the well characterized function of NCAM in multiple facets of neuroplasticity. For a more detailed overview of cell adhesion molecules in the brain see Sandi, (2004) and Dalva et al., (2007).

1.7.1 Polysialylated neural cell adhesion molecule

The neural cell adhesion molecule (NCAM) is a member of the immunoglobulin superfamily of cell adhesion molecules and serves to mediate $Ca2^{+}$-independent cell-cell and cell-extracellular matrix (ECM) interactions (Bonfanti, 2006). Through homo- and heterophilic interactions, NCAM functions in cell migration, neurite outgrowth and targeting, axonal branching,

synaptogenesis, and synaptic plasticity (Bonfanti, 2006; Bonfanti and Theodosis, 2009; Senkov et al., 2012). Neural plasticity mediated through the NCAM protein is facilitated through post-translational modifications, the most important and prevalent of which is glycosylation with polysialic acid (PSA) (Finne et al., 1983). Polysialic acid is a linear homopolymer of α2,8-linked sialic acid, which bears a negative charge, acting to abate NCAM-NCAM interactions and therefore interfere with cell adhesion (Gascon et al., 2007a). PSA-NCAM serves to regulate cell-cell and cell-ECM interactions during times of plasticity. The polysialic acid chain is added to the NCAM protein via two polysialtransferases: PST (also known as ST8SiaIV) and STX (also known as ST8SiaII). Both enzymes are individually sufficient to synthesize and attach PSA chain to an NCAM protein (Rutishauser, 2008). However, while both PST and STX are expressed throughout development, on PST is present in the adult brain (Close et al., 2001). The length of PSA chains varies, with no specific termination or capping event it seems that the length of the chain is determined by reduced binding affinity as the chain becomes longer, where chains of dozens of sialic acid residues have been observed (Livingston et al., 1988). The polysialtransferases localize to the Golgi apparatus within the cell, but are also expressed at the cell surface and in the extra cellular space (Close et al., 2001).

Figure 1.5: Diagram of a potential mechanism by which PSA-NCAM could facilitate activity-dependent neuroplasticity (top), and the inhibition of this plasticity by EndoN treatment via the cleavage of the PSA moiety (bottom).



In the adult brain PSA-NCAM is expressed on the cell surface of newly generated daughter cells, on neurites during outgrowth and path finding, and at the synapse of mature neurons (Bonfanti, 2006). The addition of the PSA moiety to NCAM is essential to neural remodelling and synaptic plasticity (Rutishauser et al., 1985; Muller et al., 1996; Burgess et al.,

2008) (see Figure 1.5). Selective cleavage of PSA in the adult brain inhibits activity-induced synaptic plasticity (induction of long-term potentiation (LTP) and long-term depression (LTD)) and alters the normal migration and integration of newly generated neurons within the hippocampus (Muller et al., 1996; Burgess et al., 2008). Importantly though, cleavage of PSA from NCAM does not disrupt normal basal synaptic neurotransmission or alter normal levels of neural proliferation or survival (Muller et al., 1996; Burgess et al., 2008). PSA-NCAM is therefore a particularly interesting protein in that it is one that mediates plasticity at multiple levels, including neural proliferation, integration, differentiation, neuritic outgrowth, synaptogenesis and the modulation of mature synapses.

Table 1.2: Selected publications delineating the role of PSA-NCAM in adult hippocampal neurogenesis.

Authors	Species	Role in neurogenesis	Ablation method	Findings
McCall et al. 2013 [97]	Rat	Neurite outgrowth; survival	EndoN	Cleavage of PSA-NCAM caused expanded dendritic arborization and increased cell death
Burgess et al. 2008 [10]	Rat	Migration; differentiation	EndoN	Cleavage of PSA-NCAM disrupts normal migration and differentiation of newly generated neurons in the DG
Seri et al. 2004 [99]	?	Differentiation	—	PSA-NCAM is highly expressed in the entire cell body and growing processes of D cells (precursors in the generation of new granule neurons in the dentate gyrus)
Ni Dhuill et al. 1999 [100]	Human	Proliferation; neurite outgrowth	—	Hippocampal expression of PSA-NCAM throughout life in humans closely resembles that of the rat. Expression largely contained to granule cells of the dentate gyrus and their mossy fiber axons, with large reductions in expression with age
Seki and Rutishauser 1998 [98]	Mouse	Neurite outgrowth	NCAM KO; EndoN	Aberrant collateral sprouting of mossy fibers and ectopic synaptic bouton formation
Kuhn et al. 1996 [101]	Rat	Migration	—	Age related decline in PSA-NCAM expression in the GCL, reduced migration of PSA-NCAM expressing cells into the GCL
Fox et al. 1995 [102]	Rat	Proliferation	—	PSA-NCAM expression decreases with age, coinciding with decreased cell proliferation
Seki and Arai 1993 [103]	Rat	Proliferation; migration; differentiation	—	Newly generated granule cells in the dentate gyrus express a highly polysialylated form of NCAM, involved in the migration of immature neurons from the subgranular zone into the GCL
Seki and Arai 1991 [104]	Rat	Proliferation	—	Highly polysialylated form of NCAM is persistently expressed in the adult dentate gyrus

EndoN: endoneuraminidase N; GCL: granule cell layer.

1.7.2 Polysialylated neural cell adhesion molecule, stress, and depression

The expression of PSA-NCAM is altered by stress and depression in a manner that coincides with the stress- and depression-induced alterations in neuroplasticity. Depressed patients show reduced expression of the soluble isoform of NCAM in their cerebrospinal fluid (CSF) compared to healthy controls (Jorgensen, 1988), while also showing alterations in PSA-NCAM expression within limbic structures. PSA-NCAM expression is reduced in the amygdala of patients with major depressive disorder (Varea et al., 2012; Maheu et al., 2013), however no significant change is seen in the PFC (Gilabert-Juan et al., 2012). Importantly, no study has assessed the influence of antidepressant treatment on PSA-NCAM expression. It is therefore important to assess the alterations to PSA-NCAM expression resulting from exposure to stress and antidepressant treatment in animal models.

Reductions of PFC and hippocampal volume induced by CUS exposure coincide with reduced NCAM expression in both regions. Conversely, chronic antidepressant treatment, with either a TCA or a SSRI, serves to enhance PFC and hippocampal volume while increasing the expression of NCAM in CUS exposed rats (Bessa et al., 2009). Expression of PSA-NCAM is also decreased in the hippocampus, amygdala and PFC in animal models of depression; while chronic antidepressant treatment enhances PSA-NCAM levels in each of these areas (Sairanen et al., 2007; Varea et al., 2007a; Wainwright et al., 2011). Perhaps most importantly, the genetic knockout of NCAM ($NCAM^{-/-}$) induces a depressive-like phenotype in rodents, an effect not observed in the ablation of hippocampal neurogenesis or the depletion of monoaminergic neurotransmission (Santarelli et al., 2003; Aonurm-Helm et al., 2008; Bessa et al., 2009; David et al., 2009; Angoa-Perez et al., 2014).

It is important to emphasize the role of PSA-NCAM in adult hippocampal neurogenesis, as PSA-functions in cell proliferation, migration, differentiation, and survival; therefore changes in the polysialylation of newly generated neurons alters neurogenesis as a whole (Seki and Arai, 1991, 1993; Fox et al., 1995; Kuhn et al., 1996; Ni Dhuill et al., 1999; Seri et al., 2004) (see Table 1.2). For instance, CUS reduces the expression of both the core NCAM protein (Bessa et al., 2009) and the addition of the PSA moiety (Sandi et al., 2001; Pham et al., 2003; Nacher et al., 2004b; Cordero et al., 2005; Wainwright et al., 2011) in the hippocampus; therefore alterations in behaviour and antidepressant efficacy may, at least in part, be mediated through PSA-NCAM. However, the expression of PSA-NCAM in depressed patients, in animal models of depression, and in response to antidepressant treatment, is dependent on both the region of the brain assessed, and the type of stressor applied in the model of depression (see Table 1.3).

The expression of PSA-NCAM is also influenced by monoamines. The expression of NCAM is directly modulated by the 5-HT1_A receptor (Grzegorzewska et al., 2010), and serotonergic innervation modulates PSA-NCAM expression within the whole hippocampal formation (Brezun and Daszuta, 2000). Moreover, chronic antidepressant treatment reduces PSA-NCAM expression in the dorsal raphe nucleus (Homberg et al., 2011), implicating PSA-NCAM in antidepressant-induced plasticity related to serotonergic neurotransmission. Similarly, the expression PSA-NCAM in the hippocampus is modulated by norepinephrine (Rizk et al., 2006), while PSA-NCAM expression in the PFC is modulated by dopamine via the D_2 receptor (Castillo-Gomez et al., 2008). While these studies do not make concrete associations between PSA-NCAM and the monoaminergic mechanisms of antidepressant action, it is important to note that plasticity resulting from the modulation of monoaminergic tone may, at least in part, be dependent upon PSA-NCAM.

Table 1.3: Effects of stress and depression on the expression of PSA-NCAM.

(a) Animal models of stress and depression

Reference	Species	Sex	Model	Antidepressant	Effect on PSA-NCAM
Gilabert-Juan et al. 2012 [110]	Mouse	M	Chronic restraint stress	—	↔ in mPFC
Djordjevic et al. 2012 [111]	Rat	M	Chronic social isolation	—	↑ in HPC; ↓ PFC
Djordjevic et al. 2012 [109]	Rat	M	Chronic social isolation	Fluoxetine	↑ in HPC, ↓ by Flx treatment
Djordjevic et al. 2012 [112]	Rat	M	Chronic social isolation	Fluoxetine	↑ in PFC; ↓ by Flx treatment (with stress)
Gilabert-Juan et al. 2011 [113]	Mouse	M	Chronic restraint stress	—	↓ in CeM; ↔ in BLA; ↔ Me
Wainwright et al. 2011 [11]	Rat	M	Unpredictable chronic mild stress	—	↓ in HPC
Homberg et al. 2011 [116]	Rat	M	—	Fluoxetine	↓ in dRN; ↔ in mPFC; ↑ AMYG (adolescent), ↓ AMYG (adult)
Varea et al. 2007 [117]	Rat	M	—	Fluoxetine	↑ in HPC (str. luc. only); ↓ in Me and BMA; ↔ in BLA
Sairanen et al. 2007 [118]	Rat	M	—	Imipramine	↑ in HPC; ↑ plPFC
Varea et al. 2007 [119]	Rat	M	—	Fluoxetine	↑ in mPFC (whole); ↑ ilPFC; ↔ plPFC
Cordero et al. 2005 [105]	Rat	M	Chronic restraint stress	—	↓ in CeM; ↓ Me
Nacher et al. 2004 [66]	Rat	M	Oral corticosterone administration	—	↓ in HPC
Nacher et al. 2004 [106]	Rat	M	Chronic restraint stress; oral corticosterone administration	—	↓ in piriform cortex (oral CORT); ↑ in piriform cortex (restraint)
Pham et al. 2003 [107]	Rat	M	Chronic restraint stress	—	↑ in HPC (3 weeks), ↔ in HPC (6 weeks)
Sandi et al. 2001 [108]	Rat	M	Chronic restraint stress	—	↑ in HPC

(b) Human studies of depression

Reference	Subjects	Sex	Population assessed	Antidepressant	Effect on PSA-NCAM
Maheu et al. 2013 [114]	Human	?	Depressed patients postmortem	Specific classes not disclosed	↓ in BLA
Gilabert-Juan et al. 2012 [115]	Human	M and F	Depressed patients postmortem	Specific classes not disclosed	↔ in dlPFC
Varea et al. 2012 [2]	Human	M and F	Depressed patients postmortem	Specific classes not disclosed	↓ in BLA; ↓ in BMA

Me: medial amygdala; CeM: centromedial amygdala; BMA: basomedial amygdala; dRN: dorsal raphe nucleus; str. luc.: stratum lucidum; plPFC: prelimbic cortex; ilPFC: infralimbic prefrontal cortex; HPC: hippocampus; AMYG: amygdala; mPFC: medial prefrontal cortex.

PSA-NCAM also interacts with BDNF, as enzymatic removal of PSA from NCAM inhibits the induction of LTP, while the application of exogenous BDNF restores LTP at the affected synapse (Muller et al., 2000). Further, disruption of the polysialylation of NCAM also disturbs the effects of BDNF on cortical neuron differentiation and survival, while the application of exogenous BDNF reverses these effects (Vutskits et al., 2001). These findings suggest that PSA-NCAM may mediate the responsiveness of neurons to BDNF. It is known that

PSA-NCAM interacts with, and may regulate, expression of the p75 BDNF receptor in septal neurons (Burgess and Aubert, 2006) and newly generated neurons of the SVZ (Gascon et al., 2007b). Indeed, knockout of p75 significantly reduces the expression of PSA-NCAM in SVZ neuroblasts (Young et al., 2007). Importantly, the p75 receptor is involved in the regulation of adult hippocampal neurogenesis (Catts et al., 2008) and regulates neurogenesis stimulated by chronic antidepressant treatment (Colditz et al., 2010).

Taken together these findings suggest PSA-NCAM may play a fundamental role in mediating broad effects of antidepressant treatment via multiple mechanisms and across multiple forms of neuroplasticity. It then stands to reason that the modulation of PSA-NCAM may be an interesting target for pharmacological antidepressant intervention. Chapter 4 aimed to assess the importance of PSA-NCAM expression to the efficacy antidepressant treatment, thereby identifying a novel mechanism through which antidepressant drugs elicit their effect, and demonstrating that neuroplasticity is not simply a bi-product of other antidepressant mechanisms.

1.7.3 Endoneuraminidase N

An enzymatic tool to manipulate the polysialation, and therefore the plasticity mediated through PSA-NCAM, exists in endoneuraminidase N (EndoN). EndoN is a soluble enzyme that is produced by the bacteriophage K1F to cleave the carbohydrate capsule (made up of polysialic acid) surrounding K1 antigen-positive serotypes of *Escherichia coli* (E. coli) (Vimr et al., 1984). EndoN serves to rapidly and specifically degrade polysialic acid homopolymers of at least 7-9 residues with α2,8-ketosidic linkages (Rutishauser et al., 1985). Application of EndoN in the adult brain inhibits activity-induced synaptic plasticity (induction of LTP and LTD) without disrupting basal synaptic transmission, produces enhanced dendritic arborization, aberrant mossy

fiber sprouting, and ectopic synaptic bouton formation (Muller et al., 1996; Seki and Rutishauser, 1998; McCall et al., 2013). EndoN treatment also alters the normal migration and integration of newly generated neurons within the hippocampus but does not alter normal levels of neural proliferation or survival (Burgess et al., 2008). Chapter 4 describes the use of EndoN to ablate PSA-NCAM and assess the effects on antidepressant efficacy within a CUS model of depression.

1.8 Thesis Overview and Objectives

The experiments described in this thesis investigate the neurobiological correlates in the pathogenesis and treatment of stress-induced depressive-like endophenotypes, with a specific focus on hippocampal neuroplasticity. Furthermore, we aimed to assess the role of androgens in conferring resiliency in the development of depressive-like endophenotypes, and the potential antidepressant action of testosterone, alone or in conjunction with a TCA, within a CUS model of depression. Finally, we sought to elucidate the role of PSA-NCAM in the antidepressant efficacy. The overarching hypothesis of the thesis is that androgens confer resiliency to the development of depressive-like phenotypes and produce antidepressant-like effects through the enhancement of neuroplasticity, which is essential to the antidepressant efficacy.

Chapter 2: To determine whether gonadectomy enhances the development of depressive-like endophenotypes, including reductions in hippocampal neurogenesis and neuroplasticity, in a chronic unpredictable stress model of depression in adult male rats.

We hypothesized that in the absence of gonadal hormones male rats would develop potentiated physiological, endocrine, behavioural, and neurological depressive-like endophenotypes,

compared to intact males. Specifically, we expected to see greater reductions in neurogenesis and the expression of PSA-NCAM corresponding with increased depressive-like behaviours in rats that are both gonadectomised and exposed to CUS, than either treatment alone.

Chapter 3: To determine whether treatment with testosterone following gonadectomy produces antidepressant-like effects, alone or in conjunction with an antidepressant drug, and a corresponding enhancement of neuroplasticity in a chronic unpredictable stress model of depression in adult male rats.

We hypothesized that testosterone treatment would reduce depressive-like endophenotypes in gonadectomised male rats exposed to CUS, both alone and in conjunction with the tricyclic antidepressant drug, imipramine. Furthermore, we hypothesized that the testosterone-induced reductions in depressive-like endophenotypes in would correspond with enhanced hippocampal neurogenesis and PSA-NCAM expression.

Chapter 4: To determine whether neuroplasticity mediated through the polysialylated form of the neural cell adhesion molecule is required for antidepressant efficacy in a chronic unpredictable stress model of depression in adult male rats.

We hypothesized that the enzymatic depletion of PSA-NCAM by EndoN would inhibit the antidepressant effects of the selective serotonin re-uptake inhibitor fluoxetine, by preventing the enhancement of hippocampal neuroplasticity.

2. HYPOGONADISM PREDISPOSES MALES TO THE DEVELOPMENT OF BEHAVIOURAL AND NEUROPLASTIC DEPRESSIVE PHENOTYPES.[1]

2.1 Introduction

The incidence rate of depression is 2-3 times greater in women than men, with this sex difference most pronounced during reproductive years (Gutierrez-Lobos et al., 2002). While several psychosocial theories regarding this sex difference do exist the prevailing neurobiological hypothesis suggests that gonadal hormones may play a substantial role in mediating the presentation and progression of depression (Hammarstrom et al., 2009). For example lower levels of testosterone in young and older men are associated with an increase in the occurrence of depression-spectrum disorders (Shores et al., 2004; Shores et al., 2005; McIntyre et al., 2006; Veras and Nardi, 2010) and androgen replacement therapy can be efficacious in the treatment of depression in hypogonadal men (Vogel et al., 1978; Seidman and Rabkin, 1998; Shores et al., 2009; Zarrouf et al., 2009). Consequently androgens may have antidepressant properties which could impede the development of, or ameliorate, extant depressive disorders.

In the current study we wished to examine the effects of surgical hypogonadism on the development of depressive-like phenotypes in an animal model of depression. Depression is a heterogenous disorder involving complex cognitive and emotional processes which are difficult to model in animals (Anisman and Matheson, 2005).

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[1]Wainwright SR, Lieblich SE, Galea LA (2011) Hypogonadism predisposes males to the development of behavioural and neuroplastic depressive phenotypes. *Psychoneuroendocrinology* 36:1327-1341.

Animal models of depression, while not modelling all symptoms of depression, can reproduce endophenotypes which can be evaluated independently and in concert at behavioural and neurophysiological levels (Hasler et al., 2004). Most models of depression use chronic exposure to stress or administration of glucocorticoids as means of generating the disrupted neuroendocrine function, neurophysiological and behavioural phenotypes.

Stress is cited as the leading cause of depression by depressed patients (Schule, 2007) and depressed patients show abnormal hypothalamic-pitutary-adrenal (HPA) axis function such as hypersecretion and abnormal diurnal secretion of cortisol resulting in a flattened rhythm (Parker et al., 2003; Schule, 2007). In fact, dysregulation of the HPA system is the most prominent endocrine change seen with depression and normalizing the HPA axis is one of the major targets of recent therapies (Keck, 2006; Schule, 2007). Interestingly, a close interaction exists between the HPA axis and the hypothalamic-pituitary gonadal (HPG) axis, where testosterone aids the regulation of basal and stress-mediated HPA function (Viau, 2002; Seale et al., 2004b).
Using an animal model of depression allows us to examine the neural characteristics of depression including alterations within the hippocampus. The hippocampus contains an abundance of glucocorticoid receptors and is particularly vulnerable to the effects of stress and has been implicated in depression (Raone et al., 2007). Depressed patients show reductions in hippocampal volume (Sheline et al., 1996; Sheline et al., 2003; McKinnon et al., 2009), gray matter density and neuropil (Stockmeier et al., 2004; Hercher et al., 2009). These alterations in hippocampal volume, structure and density could be due to a number of factors such as reductions in cell volume (Sapolsky et al., 1985b), synaptic complexity (Foy et al., 1987; Bessa et al., 2009) and/or neurogenesis (Gould et al., 1997; Gould et al., 1998).

Hippocampal neurogenesis is reduced in depressed patients and conversely neural progenitor cell production is upregulated with antidepressant treatment (Boldrini et al., 2009). Similarly, animal models of depression result in reduced hippocampal neurogenesis (Gould et al., 1998; Green and Galea, 2008; Brummelte and Galea, 2010b) and exposure to chronic, but not acute, antidepressants serves to increase cell proliferation and neurogenesis (Malberg et al., 2000). Although decreased neurogenesis has been observed in animal models of depression it does not always follow that reduced neurogenesis alone is associated with depressive-like behaviours (Santarelli et al., 2003; Airan et al., 2007; Holick et al., 2008). However, it has been suggested that the upregulation of neurogenesis or increased neural remodelling is essential to the efficacy of antidepressant drugs and the alleviation of a depressive behavioural phenotype (Santarelli et al., 2003; Bessa et al., 2009).

There is emerging evidence that reductions in synaptic complexity and diminished expression of neuroplastic proteins, such as polysialated neural cell adhesion molecule (PSA-NCAM), correlate highly with the expression of a depressive phenotype in animal models (Nacher et al., 2004a; Sairanen et al., 2007). PSA-NCAM is essential for proper neurite outgrowth (Doherty et al., 1990), axonal growth (Zhang et al., 1992; Seki and Rutishauser, 1998), synaptic plasticity (Becker et al., 1996; Muller et al., 1996), neural migration (Ono et al., 1994), the survival (Vutskits et al., 2006) and proper differentiation and integration (Burgess et al., 2008) of newly formed neurons within the hippocampus. Given its role in hippocampal neurogenesis and synaptic plasticity, alterations in PSA-NCAM expression may occur with the development of depression. Accordingly chronic unpredictable stress or chronic administration of corticosterone reduces expression of PSA-NCAM and NCAM while chronic administration of

antidepressant drugs enhances expression of PSA-NCAM within the hippocampus (Nacher et al., 2004a; Sairanen et al., 2007; Bessa et al., 2009).

Interestingly, androgens modulate both hippocampal neurogenesis and hippocampal synaptic plasticity (Leranth et al., 2003; Spritzer and Galea, 2007). Both testosterone and its metabolite dihydrotestosterone (DHT) enhance hippocampal neurogenesis through cell survival via an androgen-dependent mechanism in the dentate gyrus (Spritzer and Galea, 2007). Moreover, testosterone and DHT maintain normal synaptic density (MacLusky et al., 2006) and increase dendritic spine density (Hatanaka et al., 2009) in the hippocampus but to date no one has studied the regulation of PSA-NCAM with testicular hormones. Given the relationship between androgens and hippocampal neuroplasticity and the association of decreased androgen levels to the presentation of clinical depression it is possible that alterations in androgen levels may affect the pathoetiology of depression.

Animal models of depression often use exposure to stress or administration of exogenous corticosterone (CORT) to produce depressive-like phenotypes. For example, chronic administration of stressors or CORT is seen to produce depressive symptomology within a number of behavioural measures including the Forced Swim Test (Kompagne et al., 2008) and reduce hippocampal neurogenesis (Santarelli et al., 2003; Brummelte and Galea, 2010a, b) as well as expression of PSA-NCAM (Nacher et al., 2004a). Furthermore, neurogenic and neuroplastic changes are not mutually exclusive, as PSA-NCAM appears essential to the proper, migration, integration and differentiation of neural progenitor cells within the hippocampus (Burgess et al., 2008).

In this study we examined the effect of castration on the development of depressive behavioural and neurophysiological phenotypes in a rodent model of depression. We sought to

determine whether the absence of testicular hormones predisposes subjects to the development of depressive behavioural, neurogenic and neuroplastic phenotypes when exposed to chronic unpredictable stress. Given previous findings that show gonadectomy and chronic unpredictable stress exposure to independently reduce hippocampal neurogenesis we hypothesized that in combination these treatments will result in a potentiated decrease in cell proliferation, neurogenesis and PSA-NCAM in the hippocampus and a potentiated increase in depressive-like behaviour.

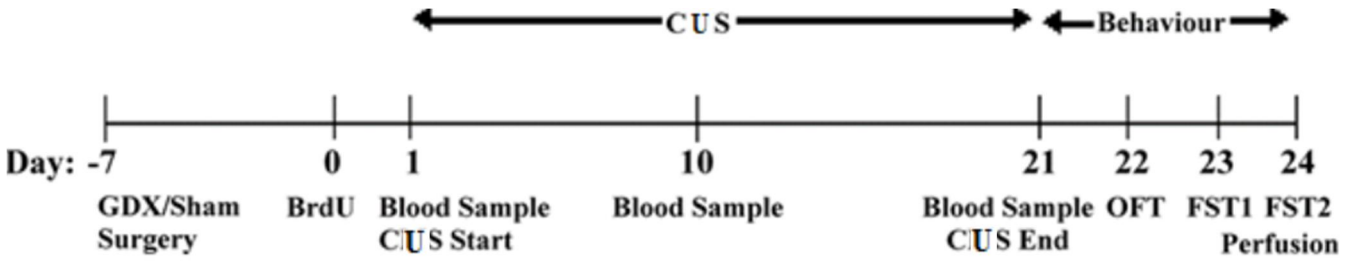
2.2 Methods

2.2.1 Subjects

Thirty-four male Sprague-Dawley rats were obtained from the Animal Care Center at the University of British Columbia. All rats weighed between 225-250g (approximately 50-52 days old) upon arrival. Rats were pair-housed in standard cages with cedar bedding, paper towels, and a polyvinylchloride tube in a temperature-controlled room [(21±1)ºC] with a 12:12 h light/dark cycle (lights on at 0700 h). After arrival the rats were given a week to habituate to their new environment. Food (Purina rat chow) and water were provided *ad libitum*, except during periods of deprivation associated with the stressing protocol. Rats were randomly assigned to into four groups according to whether they were gonadectomised (GDX) and/or received chronic unpredictable stress (CUS): (1) Sham + no CUS; (2) Sham + CUS; (3) GDX + no CUS; (4) GDX + CUS. All testing and procedures were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of

British Columbia. All efforts were made to reduce the number of animals used and to minimize their suffering.

Figure 2.1: Experimental timeline of procedures and behavioural measures.



2.2.2 Surgery

Surgeries were conducted two weeks after rats arrived in the colony using aseptic procedures, under isoflurane anesthesia (5% in oxygen during induction, 3% in oxygen during maintenance). Males were randomly assigned to receive either bilateral castration or sham-castrations. For castrations, both testes were extracted through a small incision made at the posterior tip of the scrotum, were ligated with a monofilament suture and removed. Sham operations involved incisions into the skin and muscle layers of the scrotum that were sutured without removing the testes. Immediately after surgery, Flamazine cream (1% silver sulfadiazine) was applied to the incision site and each rat was given an s.c. injection of Ketoprofen (5 mg/kg body mass) as analgesics. After surgery, rats were singly housed and one week was allowed for full recovery from surgery prior to further experimentation.

2.2.3 BrdU administration

Twenty-four hours prior to the beginning of CUS or cage handling all rats were given an i.p. injection of the thymidine analog BrdU (5-Bromo-2-deoxyuridine; 200 mg/kg) to label dividing cells and their progeny. The timing of BrdU injection relative to CUS was chosen so that we could examine the effects of CUS on neurogenesis independent of the ability of CUS to alter cell proliferation. BrdU (Sigma-Aldrich, St. Louis, MO, USA) was prepared prior to injection by dissolving 20 mg/mL in warm 0.9% saline buffered with 0.7% 1*N* NaOH.

2.2.4 Chronic unpredictable stress

Following recovery from GDX or sham surgery rats from each group were randomly assigned into two further groups and were either subjected to 21 days of CUS or acted as cage controls and were handled twice weekly (Fig.1). Cage control rats were housed in a separate colony room from rats receiving CUS in order to eliminate any potential confounds of proximity to stressed conspecifics. The CUS paradigm was adapted from that previously described by Jayatissa et al. (2006). The stress protocol consisted of the pseudo-random presentation of two stressors/day spaced at least two hours apart; the applied stressors are outlined in Table 2.1. Body weight for each rat was determined on days 1, 10 and 21 during the application of CUS to assess general physiological impact of stress administration. Body weight was measured as the percentage change in weight from day 1 to days 10 and 21 for each rat.

Table 2.1: Stressors applied pseudo-randomly over 21 days as part of CUS.

Stressor	Description
Restraint	Animals restrained in transparent Plexiglas tubes for 1 h and returned to original cage afterward.
White Noise	Animals exposed to white noise (80 dB) for 2 h.
Stroboscopic light	Stroboscopic light (~1 flash/s) for 1 h.
Wet bedding	2 h with wet bedding (700 ml water in 350 g cage bedding). Animals placed into clean dry cage afterward.
Platform	Animals placed on 20 × 20 cm transparent Plexiglas platforms<comma> mounted on 90 cm high posts<comma> for 5 min. Animals returned to original cage afterward.
Water deprivation	18 h of water deprivation immediately followed by 1 h exposure to an empty bottle.
Food Deprivation	18 h of food deprivation immediately followed by 1 h of restricted access to food (5 pellets).
Social isolation	Animals singly housed for 18 h in small cages with a thin layer of bedding. Animals returned to original cage afterward.
Cage Tilt	Home cages tilted to a 30° angle for 2 h. without water bottles.
Soiled cage	Animals placed in a cage with soiled bedding from other animals for 1 h. Animals placed in clean cage afterward.
Tail Bleed	Blood taken from tail vein, animals then placed in a new cage.

2.2.5 Open field test

In order to assess activity level and anxiety-like behaviour, the open field test was used on experimental day 22 (Fig. 1) as previously described (Galea et al., 2001; Brummelte et al. 2006). Briefly, the apparatus, a 120×120 cm2 arena divided into 16 squares of equal dimension with 40 cm high walls, was placed in a dimly lit room surrounded by an opaque curtain used to block all visual clues. Testing began 24h following administration of the final stressor, all rats were placed individually in the field facing a corner and locomotor activity was video recorded for a 10 min period. An observer blind to conditions scored the number of squares, peripheral or central, entered by the animals (an entry was defined as all four paws in a quadrant), the total number of squares entered and the number of fecal boli from each rat. Additionally the program Any-maze (Stoelting, Wood Dale, IL) was used to automatically collect the total distance travelled within the field and the percentage of time each rat spent exhibiting thigmotaxic behaviour, defined as the time spent in contact with one of the exterior walls of the open field. The apparatus was wiped with 70% ethanol thoroughly between animals.

2.2.6 Forced swim test

To assess depressive-like behaviour in all rats, the forced swim test was used on experimental day 23, 24h following the OFT (Fig. 1), as previously described (Galea et al., 2001; Brummelte et al. 2006). The forced swim test is a widely recognized measure of behavioural despair in rodents (Porsolt et al., 1977a). Scoring of several active (swimming, diving, struggling, etc.) and passive (immobility, maintenance movements etc.) behaviours is done with the proportion of each determined; where administration of antidepressant drugs elevates the proportion of active

behaviours (Porsolt et al., 1977a; Willner, 1984), while elevated expression of passive behaviours is representative of a "depressive" behavioural phenotype (Galea et al., 2001). Briefly, the apparatus consisted of a vertical cylindrical glass container (45 × 28 cm) filled to a depth of 30 cm with tap water at 25 ± 0.5°C. Testing in the forced swim test was conducted over two sessions; Session 1 took place 24h after the administration of the OFT and lasted 15min, while session 2 commenced 24h later with rats being placed in the cylinder for 10min. Both test sessions were videotaped but only was scored by an observer blind to conditions (Papp and Wieronska, 2000). The behaviours scored in the forced swim test were: (1) swimming - movement of forelimbs and/or hind limbs in a paddling fashion which displaces the rat; (2) struggling - quick movements of the forelimbs such that the front paws break the surface of the water; (3) maintenance movements – small movements of the forelimbs and/or hind limbs serving the maintain the rats posture; (4) diving – swimming movements causing the head to move below the water's surface; (5) immobility - floating with the absence of any movement. For analysis the behavioural measures were further grouped into Passive (maintenance movements; immobility) and Active (swimming; struggling; diving) behaviours. In addition, the number of fecal boli excreted by each rat was counted and recorded during each session.

2.2.7 Hormone assays

Serum levels of testosterone and corticosterone were assayed for all rats. Blood was collected from the tail vein on days 1, 10 and 21 form the rats receiving CUS, while the cage controls had blood taken on days 1 and 21 only to avoid additional stress. Each sample was taken within 3 min of entering the colony room to avoid increases in corticosterone due to the acute stress of blood collection. Blood was also taken from the chest cavity at the time of perfusion for

testosterone assay. All blood samples were stored overnight at 4ºC following collection. Samples were centrifuged at 10,000 rpm for 15 min, and serum was decanted and stored at -20ºC. Serum testosterone and corticosterone samples were assayed in duplicate using a modified protocol from a commercially available ^{125}I radioimmunoassay kits for testosterone (MP Biomedicals, Costa Mesa, CA) and rat corticosterone (MP Biomedicals, Orangeburg, NY). The testosterone antibody cross-reacts 100% with testosterone and shows minor cross-reaction with 5-dihydrotestosterone (3.40%), 5-androstane-3β,17β-diol (2.2%) and 11-oxotestosterone (2%), but does not cross-react with progesterone, estrogen, or glucocorticoids (all < 0.01%). The standard curve ED50 for testosterone was 0.457 ng⁄ml, and the lower limit of detection for this assay was 0.2 ng⁄ml. The CORT antibody cross-reacts 100% with CORT, and shows minor cross-reactivity with deoxycorticosterone (0.34%), testosterone, and cortisol (0.10%), but does not cross-react with progestins or estrogens (< 0.01%). The standard curve ED50 for CORT was 135.2 ng⁄ml, and the lower limit of detection for the assay was 7.7 ng⁄ml. The average intra-assay coefficient of variation for both the testosterone and corticosterone assays were below 10%.

2.2.8 Histology

Rats were anesthetized with a lethal dose of sodium pentobarbital (Euthanyl; Bimeda-MTC, Cambridge, ON) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde on experimental day 24 immediately following the second session of the FST. Brains were extracted and postfixed with 4% paraformaldehyde (4ºC) for 24 h. Brains were then cryoprotected with 30% sucrose in 0.1 M TBS (0.08 M Tris-HCl, 0.02 M Tris-base, 0.9% saline, pH 7.4) and stored at 4ºC until slicing. A sliding microtome was used to section the brains into

10 series of coronal tissue slices at a thickness of 40 μm. Tissue was collected throughout the entire rostral-caudal extent of the hippocampus and stored in antifreeze solution (0.05 M TBS, 30% ethylene glycol, and 20% glycerol) at -20ºC until immunohistochemical processing.

2.2.9 Immunohistochemistry

2.2.9.1 BrdU

Tissue sections were incubated at room temperature in 0.6% H2O2 for 30 min followed by three rinses in 0.1 M TBS prior to a 30-min incubation at 37˚C in 2 N hydrochloric acid. The tissue was then rinsed in 0.1 M borate buffer for 10 min (pH 8.5) followed by three more rinses in 0.1 M TBS. The tissue sections were then transferred to a primary antibody solution containing 1:200 mouse anti-BrdU (Roche, Indianapolis, IN), 3% normal horse serum, and 0.1% Triton X in 0.1 M TBS for 24 h at 4˚C on a shaker. The tissue was rinsed three times in 0.1 M TBS and was then transferred to a secondary antibody solution containing a 1:500 dilution of horse anti-mouse biotinylated antibody in 0.1 M TBS. The tissue was incubated in the secondary solution for 4 h at room temperature. Following three rinses in 0.1 M TBS, an ABC kit (Vector, Burlingame, CA) was used and prepared according to the kit instructions and tissue incubated for 1.5 h. BrdU labeling was visualized by incubating tissue in 0.02% diaminobenzidine (DAB) for 3 min. Finally, the sections were mounted on glass slides and coverslipped with permount.

2.2.9.2 Ki67

Tissue was first incubated at room temperature in 0.6% H2O2 for 30 min and was then rinsed three times for 10 min each in 0.1 M PBS. The sections were then transferred to a primary

antibody solution containing a 1:500 Rabbit anti-Ki67 monoclonal antibody (Vector, Burlingame, CA), 3% Normal goat Serum, and 0.3% Triton-X in 0.1 M PBS. Tissue was incubated in the primary solution for 24 h at room temperature on a shaker and was then rinsed three times for 10 min per rinse in 0.1 M PBS. The tissue was then incubated for 16 h at room temperature in a secondary antibody solution containing a 1:500 dilution of goat anti-rabbit (Vector, Burlingame, CA). After rinsing the tissue three times in 0.1 M PBS, the tissue was next incubated in an ABC solution (Vector, Burlingame, CA) as per the kit instructions for 2 h. The tissue was rinsed in 0.1 M PBS and was then developed with DAB for 2 min. The sections were then mounted on glass slides and were coverslipped with Permount.

2.2.9.3 BrdU/NeuN

Double labeling for BrdU and NeuN was performed to assess phenotype of the BrdU-labelled cells. A series of brain tissue was incubated at 4˚C for 24 h in mouse anti-NeuN (Millipore, Temecula, CA) diluted 1:250 in 0.1 M PBS containing 0.3% triton-X. The tissue was then rinsed three times in 0.1 M PBS and was then incubated for 16 h at 4˚C in donkey anti-mouse Alexa 488 (Invitrogen, Eugene, OR) diluted 1:500 in 0.1 M PBS. The tissue was then rinsed three times in 0.1 M PBS and was then fixed in 4% PFA for 10 min and was then rinsed twice in 0.9% NaCl for 10 min each. DNA was denatured by heating sections to 37˚C for 30 min in 2N hydrochloric acid. BrdU labeling was then carried out by incubating the tissue for 24 h at 4˚C in a 1:500 dilution of Rat anti-BrdU (AbD Serotech, Oxford, UK) in 0.1 M PBS containing 0.3% Triton-X. Following three rinses in 0.1 M PBS the tissue was then transferred to a solution containing a 1:500 dilution of Donkey anti-rat Cy3 (Jackson ImmunoResearch, Westgrove, PA) for 16 h at

4˚C. Finally, the tissue was rinsed three times in 0.1 M PBS and the sections were then mounted on glass slides and coverslipped with PVA-DABCO.

2.2.9.4 PSA-NCAM

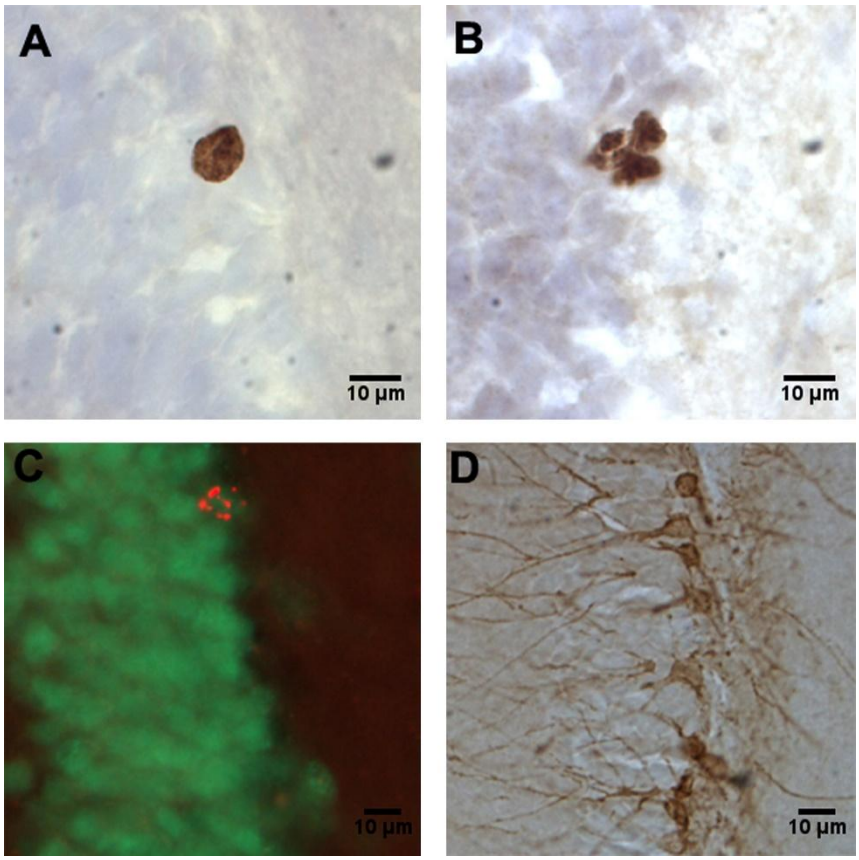
Tissue sections were incubated at room temperature in 0.3% H_2O_2 for 20 min followed by three rinses in 0.1M TBS. Sections were then transferred to a primary antibody solution containing 1:1000 mouse anti-PSA-NCAM (Millipore, Temecula, CA), 3% normal horse serum, 0.3% Triton-X in 0.1M TBS and incubated at 4˚C for 48 h on a shaker. The tissue was rinsed five times in 0.1M TBS at room temperature and was then transferred to a secondary antibody solution containing a 1:250 dilution of horse anti-mouse (Vector, Burlingame, CA) in 0.1M TBS and then incubated in the secondary solution for 24 h at 4˚C. Following five rinses in 0.1M TBS an ABC kit (Vector) was used and prepared according to kit instructions. PSA-NCAM immunoreactive cells were visualized by incubating tissue in DAB for 6 min, and were then mounted on glass slides and coverslipped with permount.

2.2.9.5 Cell counting

All counting was conducted by an experimenter blind to condition. BrdU, Ki67 and PSA-NCAM-labelled cells were counted in every 10^{th} section throughout the entire granule cell layer (GCL), including the subgranular zone (SGZ), and the hilus (separately). The subgranular zone was defined as the 50-µm zone between the GCL and hilus. Cell counts were performed in the hilus as a control for any effects that could alter BrdU incorporation such as differences in blood brain barrier permeability. Counting was performed using a Nikon E600 light microscope under a 100x oil immersion objective lens. Cell counts were aggregated and multiplied by 10 to obtain

an estimate of the total number of labelled cells, as previously described (Kronenberg et al., 2003; Eadie et al., 2005; Epp et al., 2010). The percentage of BrdU/NeuN double-labelled cells was obtained by selecting 50 BrdU-labelled cells per brain arbitrarily from at least five sections per brain and determining what percentage of these cells also expressed NeuN. Fluorescent imaging was performed on a Nikon epifluorescent microscope under 400x magnification. Area measurements of the granule cell layer and hilus were calculated separately using the software program ImageJ (NIH). The volume of each region was estimated using Cavalieri's principle (Gundersen and Jensen, 1987). In brief, the sum of the measured areas was multiplied by the distance between measured sections (400 μm) to give the volume of the region (Epp et al., 2009; Epp et al., 2010). Cell densities for Ki67-labelled, BrdU-labelled and PSA-NCAM-labelled cells were determined by dividing total cell counts by the calculated areas for each of the respective regions (GCL+SGZ and hilus). Figure 2 displays representative photomicrographs of Ki67 Ki67-labelled, BrdU-labelled and PSA-NCAM-labelled cells.

Figure 2.2: Photomicrographs of (A) BrdU-labelled (×1000 magnification), (B) Ki67-labelled (×1000 magnification), (C) BrdU/NeuN-colabelled (×400 magnification) and (D) PSA-NCAM labelled cells (×400 magnification). Scale bar represents 10 µm.



2.2.10 Data analyses

Percent change in body weight was analyzed using a repeated-measures analysis of variance (ANOVA) with stress treatment (no CUS, CUS) and hormone (Sham, GDX) as the between-subjects factor and days (1, 10, and 21) as the within-subjects factor. Analysis of the open field test and forced swim test were also measured using repeated-measures ANOVA with area of the OFT (peripheral, center) or FST behaviour (passive, active) as within-subjects factors and stress

(no CUS, CUS) and hormone (Sham, GDX) as the between-subjects factors. Serum testosterone and corticosterone levels were analysed using a repeated-measures ANOVA with day (1, 10 and 24 for testosterone; 1, 10, and 21 for corticosterone) as the within-subject factor along with stress (no CUS, CUS) and hormone (Sham, GDX) as the between-subjects factors. Volume of dentate gyrus, density of Ki67- and BrdU-labelled cells were each analysed with a repeated-measures ANOVA with area (GCL+SGZ, hilus) as within-subject factor and stress (no CUS, CUS) and hormone (Sham, GDX) as the between-subjects factors. PSA-NCAM labelled cells were analysed with a repeated measures ANOVA with and stress (no CUS, CUS) and hormone (Sham, GDX) as the between-subjects factors. All post hoc tests utilized Newman–Keuls unless otherwise specified.

2.3 Results:

2.3.1 Chronic unpredictable stress in combination with gonadectomy attenuated weight gain significantly more than either treatment alone

Both CUS and GDX produced attenuated weight gain for both day 10 and day 21 with greater weight attenuation in the GDX-CUS group than both the Sham-CUS ($p \leq 0.0002$) and the GDX-no CUS ($p \leq 0.0002$) groups (Fig. 3A; significant interaction effect of stress by hormone ($F(2,60) = 5.3$, $p \leq 0.008$; main effects of both hormone ($p<0.0001$) and stress ($p<0.0001$)).

2.3.2 Chronic unpredictable stress increased serum corticosterone in both gonadectomised and sham treatment groups

Analyses of corticosterone and testosterone were conducted using percent change from baseline on days 10 and 21. Analysis of both corticosterone and testosterone on day 10 was only carried out on CUS treated rats to avoid stressing the no-CUS groups as blood collection was used as a stressor. Thus Day 10 values for the no-CUS group were given as the same values as those collected on Day 1.

As expected there was a greater percent change in corticosterone levels in the CUS treatment groups over the no-CUS treatment groups on days 10 ($p<0.0002$) and 21 ($p<0.0002$) compared to Day 1. Figure 3B illustrates the percent-change in serum corticosterone concentration from baseline for each treatment group. There was a day by stress interaction ($F(2,40) = 10.59$, $p<0.0003$) and a main effect of stress was seen ($F(1,20) = 21.48$, $p<0.0002$) but no other significant interaction or main effects were found (all p's$\leq$0.89).

2.3.3 Chronic unpredictable stress increased serum testosterone in sham-treated groups

CUS significantly increased serum testosterone levels in the Sham group on both day 10 ($p<0.02$) and day 24 ($p\leq0.002$; stress by hormone ($F(1,19)=5.46$, $p\leq0.03$) and day by hormone ($F(2,38)=3.53$, $p<0.04$ interaction effects) (Fig. 3C). Post-hoc analyses showed that the Sham-CUS group had significantly higher testosterone levels than each of the other treatment groups: Sham-noCUS ($p<0.004$), GDX-noCUS ($p\leq0.004$) and GDX-CUS ($p\leq0.002$). There was no significant change in testosterone levels between days 10 and 24 in the Sham-CUS group ($p<0.3$). As expected, no significant change in testosterone levels were observed across days

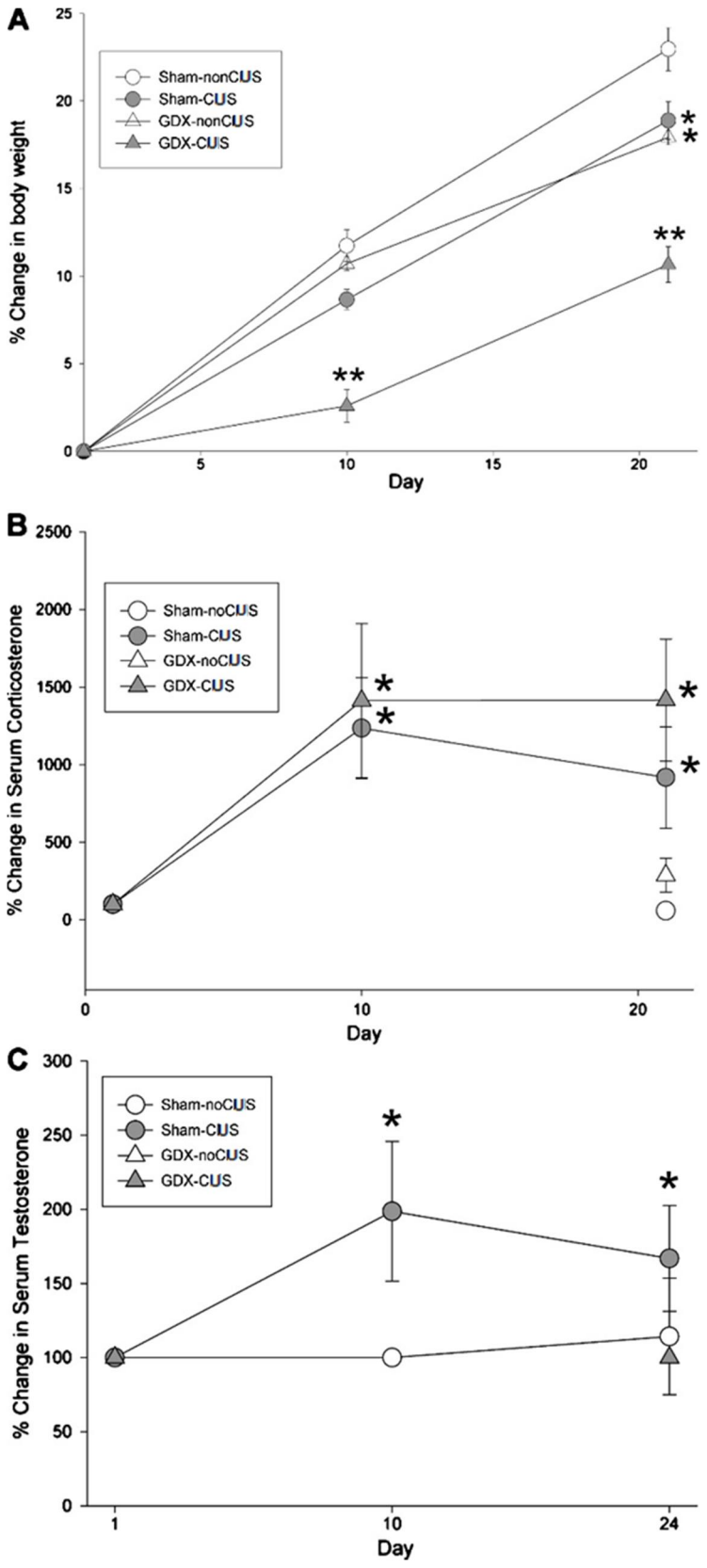
within the GDX group ($p \leq 0.99$). Furthermore, main effects of both stress ($p \leq 0.03$), and hormone ($p < 0.004$) were observed (Fig. 3C).

2.3.4 Both chronic unpredictable stress and gonadectomy increased putative measures of anxiety within the open field test

The percentage of time each rat spent displaying thigmotaxic behaviour was analyzed ((Mean ± SEM): Sham-noCUS (75.22 ± 2.49), Sham-CUS (85.99 ± 2.02), GDX-noCUS (87.96 ± 3.08), GDX-CUS (82.80 ± 1.53)). A significant stress by hormone interaction was found ($F(1,22)=11.55$, $p<0.003$), as well as a trend towards a main effect of hormone ($p<0.06$). Post-hoc analysis revealed that the Sham-CUS ($p<0.02$), GDX-noCUS ($p \leq 0.005$) and GDX-CUS ($p<0.04$) treatment groups spent significantly more time displaying thigmotaxis compared to the Sham-noCUS group.

Examination of number of crossings per area revealed that CUS resulted in differential effects on peripheral crossings in GDX versus Sham rats (Fig. 4A; interaction effect: $F(1,27)=5.91$, $p<0.02$). Post-hoc tests revealed that CUS increased peripheral crossings in GDX rats ($p<0.007$) but decreased peripheral crossings in Sham rats ($p<0.05$). There was no main effect of either stress ($p<0.9$) or hormone ($p<0.5$) on the number of peripheral or central crossings or the total number of crossings ($0.08<p<0.85$). Additionally there was no significant difference in the number of fecal boli between treatment groups ($p<0.09$).

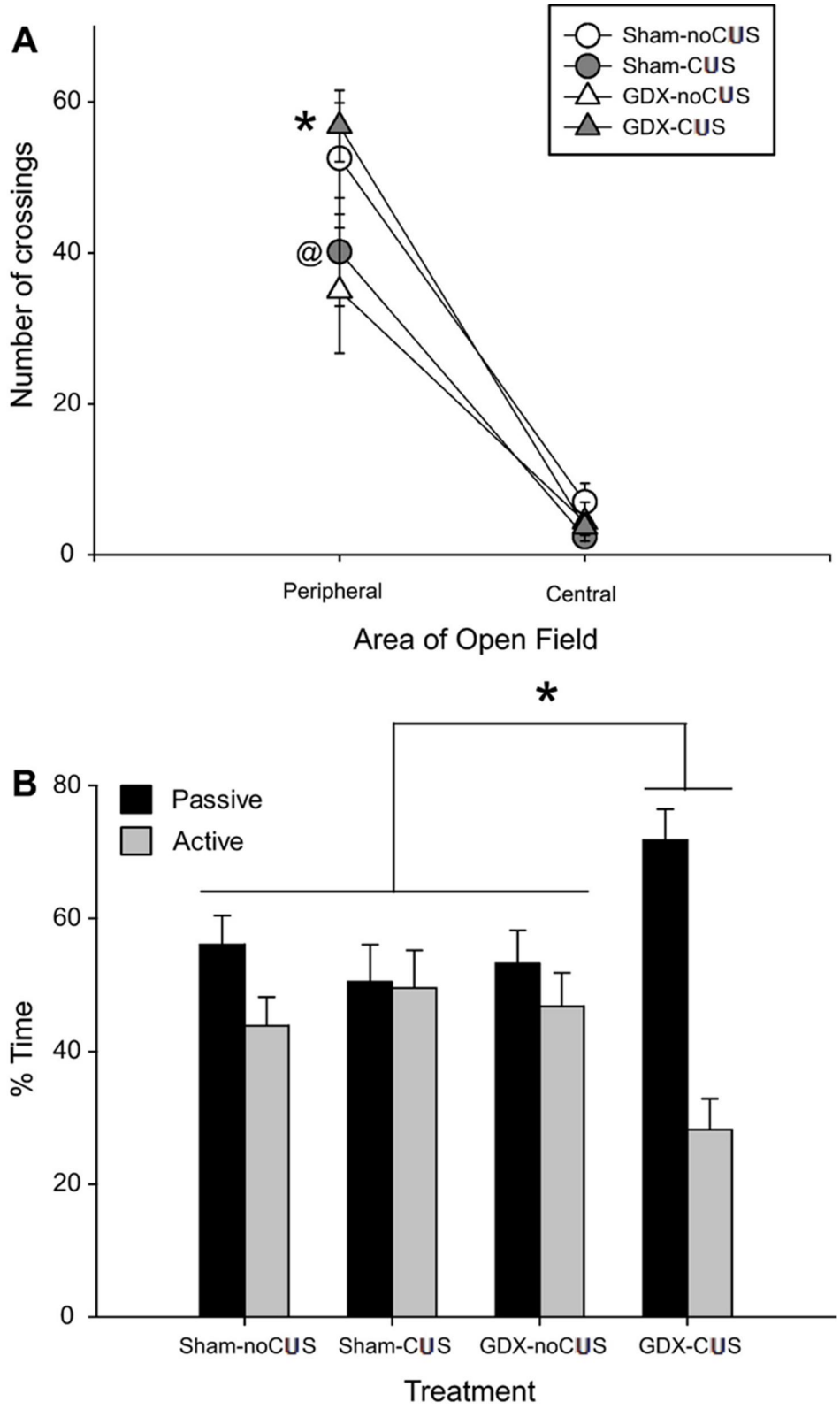
Figure 2.3: (A) Mean percent change in body weight from Day 1 on Days 10 and 21 (±SEM). Gonadectomy attenuated weight gain regardless of chronic unpredictable stress (CUS) exposure (both p's < 0.02). CUS also attenuated weight gain across the three weeks in both sham ($p < 0.02$) and GDX ($p < 0.0001$) groups. Importantly, there was greater weight attenuation in the GDX-CUS group than the Sham-CUS group ($p < 0.0001$). (B) Serum corticosterone levels significantly increased over baseline on days 10 and 21 in the CUS treatment groups (day by stress interaction ($F(2,40) = 10.59$, $p < 0.0003$)). (C) Serum testosterone levels significantly increased on days 10 and 24 over baseline in the sham males with stress (stress by hormone $p < 0.04$). As expected animals receiving GDX had significantly less testosterone than the sham groups ($p < 0.004$).


A
% Change in body weight
Sham-nonCUS
Sham-CUS
GDX-nonCUS
GDX-CUS
Day
B
% Change in Serum Corticosterone
Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
Day
C
% Change in Serum Testosterone
Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
Day

2.3.5 Gonadectomy in combination with CUS increased depressive-like behaviour in the forced swim test

Percentage of time spent engaged in either passive and active behaviours (Fig. 4B) were assessed for the second session of the forced swim test, were we found a significant behaviour by stress by hormone interaction ($F(1,25)=6.12$, $p\leq0.02$). Post-hoc analysis revealed that there was increased passive, and decreased active, behaviour in the forced swim test in the GDX-CUS group compared to all other treatment groups (all p's<0.034). As expected we also found a main effect of behaviour ($p<0.004$), with significantly more passive behaviours than active behaviours displayed. There were no other significant interactions (p's≤0.08), or main effects (p's<0.3).

Figure 2.4: (A) Mean number of crossings in the open field test (±SEM). Chronic unpredictable stress (CUS) produced opposite results on peripheral crossings in GDX versus Sham rats (stress by hormone interaction ($F(1, 30) = 5.91$, $p < 0.2$). CUS increased peripheral crossings in GDX rats ($p < 0.007$) but decreased peripheral crossings in Sham rats ($p < 0.05$). (B) Mean (±SEM) percentage of time spent immobile during the FST averaged over the two sessions of testing. GDX-CUS males spent a significantly greater period of time immobile as compared to Sham-CUS males ($p < 0.02$), measured over the two FST sessions.


A
Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
Number of crossings
60
40
20
0
*
@
Peripheral
Central
Area of Open Field
*
B
Passive
Active
% Time
80
60
40
20
0
Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
Treatment

2.3.6 There were no significant group differences on the volume of the dentate gyrus

Analysis of dentate gyrus volumes (Table 2.2) revealed, as expected, a main effect of area ($F(1,24) = 389.29$, $p \leq 0.0001$), with the hilus having a larger volume than the GCL. Stress did not produce a significant effect on dentate volumes (main effect: $p \leq 0.76$); however there was a trend for a significant main effect of hormone ($p \leq 0.06$) where GDX rats had smaller GCL volumes than Sham rats (Table 2.2). Due to this trend we chose to look at cell density scores for BrdU- , Ki67- and PSA-NCAM-labelled cells.

Table 2.2: GCL and hilus volume (mean ± SEM) in the dentate gyrus for rats within each treatment group.

Treatment	N	GCL volume (mm3)	Hilus volume (mm3)
Sham	9	3.83 ± 0.23	10.0 ± 0.74
Sham-CUS	7	3.65 ± 0.18	10.0 ± 0.56
GDX	8	3.26 ± 0.16	9.01 ± 0.47
GDX-CUS	7	3.63 ± 0.22	9.79 ± 0.76

2.3.7 CUS decreased the density of proliferating cells in the GCL only in the GDX group while GDX decreased the density of proliferating cells regardless of stress

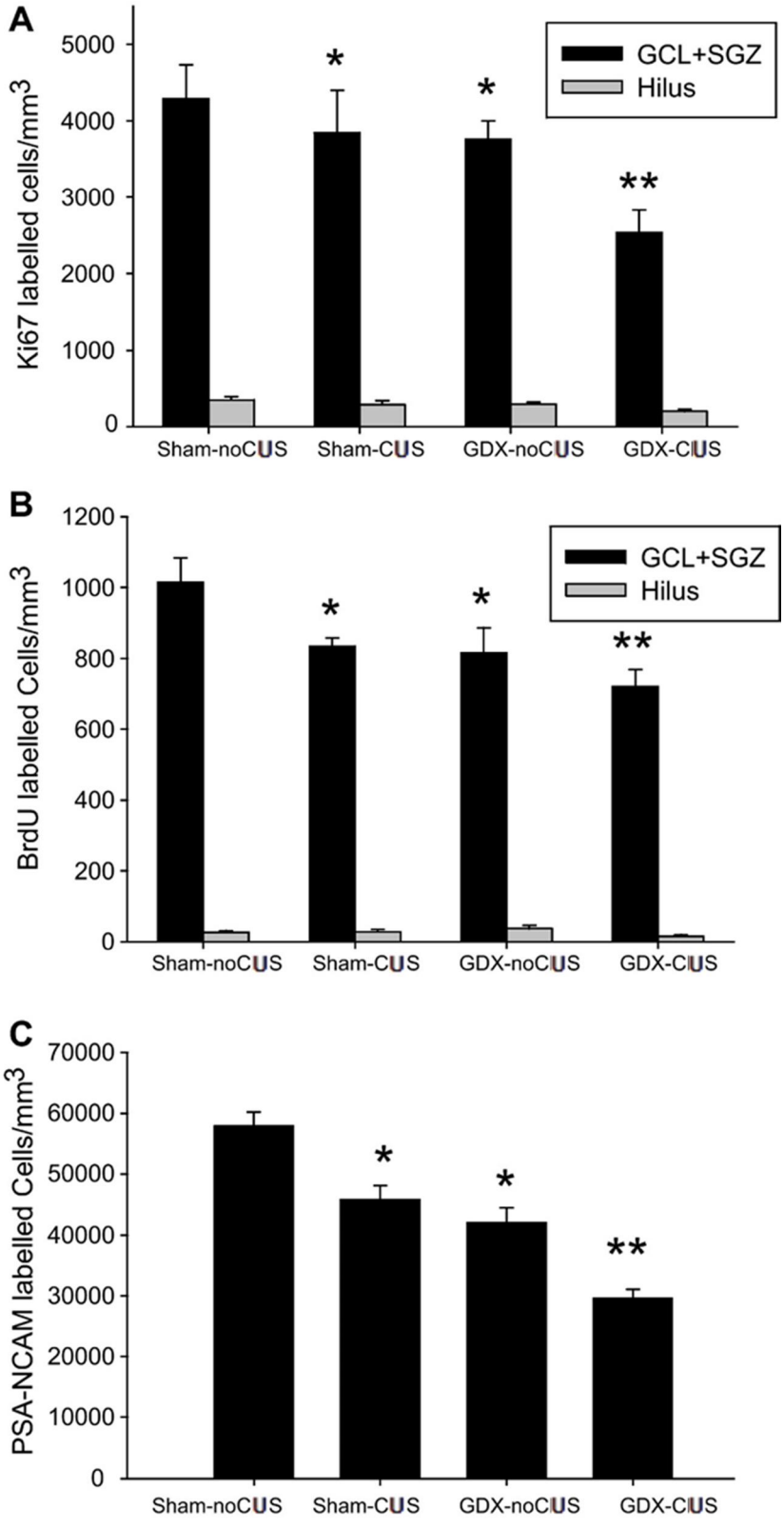
Analysis of Ki67-labelled cell density (Fig. 5A) revealed a significant stress by region interaction ($F(1,29) = 4.07$, $p \leq 0.05$) and significant hormone by region ($F(1,29) = 5.17$, $p<0.031$). Post-hoc tests revealed that CUS reduced cell proliferation within the GCL+SGZ ($p \leq 0.002$), but not the hilus ($p \leq 0.75$). Similarly, gonadectomy reduced cell proliferation within the GCL+SGZ

($p<0.0008$), but not in the hilus ($p<0.79$). We hypothesized the sham treated rats would be more resilient to stress than gonadectomised rats and thus an *a priori* test showed that CUS significantly reduced proliferation in the GDX-CUS group compared to the Sham-CUS group ($p \leq 0.0013$). Furthermore, the GDX-CUS group has significantly less proliferation than both the GDX-noCUS ($p \leq 0.0036$) and Sham-noCUS ($p<0.0001$) groups.

2.3.8 Both CUS and gonadectomy independently decrease cell survival within the dentate gyrus

Assessment of BrdU-labelled cell density (Fig. 4B) showed significant interactions of stress by region ($F(1,23) = 4.73$, $p \leq 0.04$) and hormone by region ($F(1,23) = 7.27$, $p \leq 0.013$). Post-hoc tests indicated that the density of BrdU-labelled cells was significantly reduced with CUS ($p \leq 0.003$) and gonadectomy ($p \leq 0.002$) in the GCL+SGZ but not in the hilus ($p's \leq 0.96$). There were also significant main effects of hormone ($p<0.03$) and stress ($p<0.04$) but no other significant interaction effects ($p's \leq 0.72$). We hypothesized that CUS and gonadectomy in combination would exert a greater effect on cell survival than either treatment alone. An *a priori* test showed that the GDX-CUS group had a significantly lower number of surviving BrdU-labelled cells than the Sham-CUS group ($p \leq 0.014$) and the GDX-noCUS group ($p<0.035$) within the GCL+SGZ.

Figure 2.5: (A) Mean (± SEM) density of Ki67-labelled cells in the GCL and hilus. CUS significantly reduced cell proliferation in the GCL of GDX male rats ($p < 0.003$) but not in the Sham rats ($p < 0.25$). GDX males had decreased cell proliferation in the GCL compared Shams ($p < 0.0006$). GDX-CUS also had a significant decrease in Ki67-labelled cells in the GCL compared to Sham-CUS. (B) Mean (±SEM) density of BrdU-labelled cells in the GCL and hilus. GDX-CUS males showed significantly less cell survival in the GCL compared to Sham-noCUS ($p < 0.0001$) and Sham-CUS males ($p < 0.003$) male rats. GDX, regardless of stress, decreased the density of BrdU-labelled cells in the GCL ($p < 0.032$). (C) Mean (±SEM) density of PSA-NCAM labelled cells within the GCL of the dentate gyrus, the GDX-CUS had significantly fewer immunoreactive cells than all other groups (p's < 0.02). GDX, regardless of stress, decreased the density of PSA-NCAM-labelled cells ($p < 0.005$). CUS, regardless of GDX status, decreased the density of PSA-NCAM labelled cells ($p < 0.003$).


A
Ki67 labelled cells/mm3
5000
4000
3000
2000
1000
0
GCL+SGZ
Hilus
*
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Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
B
BrdU labelled Cells/mm3
1200
1000
800
600
400
200
0
GCL+SGZ
Hilus
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Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS
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PSA-NCAM labelled Cells/mm3
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50000
40000
30000
20000
10000
0
*
*
**
Sham-noCUS
Sham-CUS
GDX-noCUS
GDX-CUS

2.3.9 CUS and gonadectomy do not affect the phenotype of newly generated cells within the dentate gyrus

There were no significant main effects of stress ($p<0.09$) or hormone ($p<0.3$), nor did we find a significant interaction (stress by hormone $p<0.9$) on the percentage of BrdU/NeuN immunoreactive cells (see Table 2.3).

2.3.10 Both gonadectomy and CUS independently decreased expression of PSA-NCAM within the dentate gyrus

Both CUS and GDX alone reduced the density of PSA-NCAM-labelling within the GCL of the dentate gyrus as there were significant main effects of both stress ($F(1,27)=11.6$, $p<0.003$) and hormone ($F(1,29)=9.86$, $p<0.005$)(Fig. 4C). There were no other significant interactions (p`s<0.5). We hypothesized the sham treatment would be more resilient to stress than the gonadectomised treatments and thus an *a priori* test showed that PSA-NCAM was significantly reduced in the GDX-CUS group compared to the Sham-CUS group ($p<0.007$).

Table 2.3: Mean (±SEM) percentage of BrdU-labelled cells that also express NeuN.

Treatment	BrdU/NeuN double-labelled cells (%)
Sham-noCUS	43.78 ± 2.25
Sham-CUS	47.43 ± 2.57
GDX-noCUS	46.50 ± 2.03
GDX-CUS	46.29 ± 2.16

2.3.11 Correlations

There was a significant positive correlation between the density of BrdU-labelled cells and the density of Ki-67-labelled ($r= 0.52$, $p\leq0.003$) and PSA-NCAM-labelled ($r= 0.45$, $p<0.02$) cells in the GCL of all treatment groups. Similarly, the number of Ki-67-labelled cells positively correlates with the number of PSA-NCAM-labelled cells ($r=0.62$, $p<0.001$). Behaviourally, the number of BrdU-labelled cells within the GCL is positively correlated to the number of central crossings within the OFT ($r=0.41$, $p<0.04$) for all groups but there were no other significant effects.

2.4 Discussion

The results described here demonstrate that the presence of testicular hormones provides resilience to the development of physiological and behavioural depressive-like phenotypes after the application of CUS. Gonadectomised male rats exposed to CUS showed enhanced development of putative markers of depression compared to intact rats exposed to CUS. Gonadectomized male rats exposed to CUS showed attenuated weight gain, enhanced serum corticosterone levels, increased passive behaviours in the forced swim test, reduced cell proliferation, cell survival and reduced density of PSA-NCAM-labelled cells in the hippocampus compared to intact males receiving CUS. Together these results suggest an increased susceptibility to developing depressive-like symptoms following stress exposure in the absence of testicular hormones. Interestingly we found that intact males demonstrated a stress-induced enhancement of serum testosterone levels with the application of CUS. Furthermore we saw that exposure to CUS for three weeks independently decreased neurogenesis, cell proliferation and the expression of the neuroplastic protein PSA-NCAM. In addition, gonadetomy alone decreased

neurogenesis and PSA-NCAM expression indicating that testicular hormones modulate these factors. Taken together our findings show that testicular hormones play an important role in the physiological response to stress, both at the cellular and systems level, which may therefore provide males with increased resilience to the development of a depressive behaviours. This study is to our knowledge the first to integrate and correlate the expression of neurogenic, neuroplastic and behavioural measures of depression and demonstrate their relation to gonadal hormone status in males.

2.4.1 The absence of testicular hormones in combination with chronic unpredictable stress potentiates reductions in weight gain while CUS increases serum corticosterone

Rats exposed to three weeks of CUS showed attenuated weight gain at both days 10 and 21, this result is consistent with previous studies using CUS (D'Aquila et al., 1994; Willner et al., 1996). Gonadectomy was also seen to independently reduce weight gain in males on both day 10 and 21 compared to intact males, a finding that coincides with the well documented effects of androgens on body weight (Bell and Zucker, 1971). Importantly males receiving both gonadectomy and CUS exposure had attenuated weight gain that was significantly greater than either treatment alone. This finding suggests an additive effect of hypogonadism and stress where in the absence of gonadal hormones the gross physiological response to stress is exacerbated. Furthermore this finding is consistent with literature showing that weight disturbance is a common feature in depressed patients, where an increased incidence rate is found in both under- and over-weight individuals (Chen et al., 2009; de Wit et al., 2009).

Exposure to CUS increased basal serum corticosterone levels on both day 10 and 21 compared to baseline levels assessed on day 1. This finding is consistent with previous research

showing that chronic unpredictable stress enhances HPA tone and increases basal corticosterone concentrations (Ayensu et al., 1995; Bielajew et al., 2002; Marin et al., 2007). Furthermore this is consistent with meta-analyses indicating that basal cortisol levels are higher, and HPA axis function is dysregulated in depressed patients (Halbreich et al., 1985; Peeters et al., 2004; Lopez-Duran et al., 2009; Vreeburg et al., 2009). In the present study no significant difference in serum CORT was noted between gonadectomised and intact rats with or without concomitant exposure to stress, a finding consistent with previous studies (Frye et al., 2010). However it is important to note that we only collected blood samples near the beginning of the light cycle, the typical nadir in the circadian rhythm of corticosterone in rats (D'Agostino et al., 1982) and thus it is possible that collection of blood at different time points may alter the results.

Exposure to chronic unpredictable stress increased serum testosterone in intact males, consistent with previous studies using repeated stress exposure in intact males (Gomez et al., 2002; Gomez et al., 2004; Gray et al., 2010). This finding suggests that in the face of stress males experience increased gonadal drive, subsequently upregulating testosterone and mitigating HPA dysregulation. Previous studies have shown the ability of testosterone to facilitate HPA habituation to repeated stressors via feedback inhibition (Retana-Marquez et al., 2003; Gray et al., 2010). Although our paradigm uses a varying unpredictable stress regime it is possible intact males acclimate better to the stress as evidenced by the non-significant decline in serum CORT levels from day 10 to 21. Additionally the effects of increased gonadal drive can be seen in the hippocampus where testicular hormones have been previously shown to facilitate neurogenesis and plasticity (Leranth et al., 2003; Spritzer and Galea, 2007) further alleviating neurophysiological depressive-like endophenotypes.

2.4.2 Exposure to chronic unpredictable stress produced contrasting effects on a putative measure of anxiety

Our findings show exposure to CUS increased thigmotaxic behavior in the open field test, and increased the number of peripheral crossings in gonadectomised male rats. Thigmotaxis is associated with heightened anxiety and is increased following exposure to stress consistent with our present findings (Archer, 1973; Katz et al., 1981; Izumi et al., 1997). In the present study we found that gonadectomy alone and in combination with CUS enhanced thigmotaxic behaviour, indicative of an anxiety-like phenotype in the open field test.

Futhermore, we found contrasting effects of GDX in combination with the CUS or noCUS treatments on thigmotaxis. In the absence of testicular hormones thigmotaxis was increased with CUS, while in intact males thigmotaxis was decreased with CUS. In the current study we did not find any treatment effects on locomotor activity which suggests our findings on thigmotaxis are not related to general motoric effects but likely are alterations in anxiety-like behaviour. Interestingly CUS was seen to increase serum testosterone levels in Sham males, an effect that may bare impact on thigmotaxis. Previous research has shown administration of testosterone metabolites increased time spent within the central region of the open field and decreased thigmotaxis in male rats (Frye et al., 2008). Moreover, endogenous elevations of testosterone in males reduced anxiety-like behaviour in the open field test (Edinger and Frye, 2007), consistent with our results. Our findings suggest that in the absence of gonadal hormones CUS induces anxiety-like behaviors. Intriguingly, we also found that CUS induces hypersecretion of testosterone in intact males which in turn has anxiolytic effects in the open field test. We however, conclude this with caution given the potential ambiguities when interpreting anxiety-like behaviour within the open field test.

2.4.3 Exposure to chronic unpredictable stress resulted in depressive-like behaviour in gonadectomized males only

The combination of gonadectomy and CUS exposure produced a significantly greater proportion of passive behaviours exhibited in the forced swim test compared to all other treatment groups. Our findings are partially consistent with past literature showing that chronic unpredictable stress amplified the expression of passive behaviours in the forced swim test (Kompagne et al., 2008). However in our study we did not observe increased passive behaviours in Sham rats receiving CUS, only the GDX-CUS group showed increased passive behaviours relative to their controls. Although some studies have found an increase in passive behaviours using the CUS protocol, others have not (Dalla et al., 2005; Liang et al., 2008), which is consistent with our findings. The inconsistency in FST results with CUS exposure is likely due to the unpredictability, type of stressors and the duration of CUS exposure. For example in the present study we gave CUS for 3 weeks but other paradigms that have found an increase in passive behavior with the FST have used at least 4 weeks of CUS exposure (Bielajew et al., 2003; Grippo et al., 2003). Although at least one study by Kompange et al.(2008) found 3 weeks of CUS to be efficacious in increasing passive behaviours within the FST, Wistar rats were used and strain differences in FST have been previously reported (Bielajew et al., 2003). Furthermore it should be noted that although we did not observe a depressive behavioural phenotype in the Sham-CUS group we did not necessarily expect one. It was our expectation that GDX in males would render this group more vulnerable to the depressive-like effects of CUS compared to the Sham group, much the same as a shortened CUS rendered females more vulnerable to a depressive phenotype compared to males (LaPlant et al., 2009).

In the present study we found that gonadectomy alone did not increase passive behaviours, a finding consistent with Frye et al. (2010). Conversely, previous studies have shown that administration of testosterone in healthy male rats serves to decrease passive behaviours within the forced swim test (Buddenberg et al., 2009). Furthermore, administration of testosterone or its metabolite DHT improves the depressive-like behavioural phenotype of aged rodents in the forced swim test (Frye and Walf, 2009). Importantly testosterone does not appear to modulate motor activity as the Buddenberg et al. (2009) study found no significant locomotor effects of testosterone administration, consistent with the findings of the present study. Therefore the increased incidence of passive behaviour with gonadectomy and CUS exposure in tandem may suggest a reduced ability to cope with chronic stress in the absence of testicular hormones; therefore castrated rats are more susceptible to developing depressive-like behaviours. However, it is important to note that this is an animal model using a single putative measure of depressive-like behaviour which may not extrapolate directly to human disease.

2.4.4 Exposure to chronic unpredictable stress and gonadectomy decreases cell proliferation within the dentate gyrus

Our present findings show exposure to chronic unpredictable stress and gonadectomy affect measures of hippocampal plasticity, both independently and in concert. A reduction in cell proliferation within the dentate gyrus was seen primarily in GDX-CUS rats compared to GDX-noCUS rats. Interestingly we found that in combination gonadectomy and CUS produced a synergistic effect on reductions in cell proliferation, where the GDX-CUS treatment group showed significantly fewer Ki67 immunoreactive cells than both the GDX-noCUS and Sham-CUS groups. This study is the first to show that hypogonadism in combination with stress

potentiates reductions in cell proliferation in the dentate gyrus. Several studies have shown significant reductions in cell proliferation within the dentate gyrus after exposure to CUS (Liu et al., 2008; Bessa et al., 2009; Jayatissa et al., 2009), consistent with the present study. However, other studies have not found such alterations in cell proliferation following CUS (Lee et al., 2006; Wu and Wang, 2010). The observed variations in the efficacy of CUS in reducing cell proliferation may be due to a number of factors including the strain of rat used, length of exposure to CUS, and the predictability of the paradigm. For example Jayatissa et al.(2009) used a CUS paradigm similar to the one used in the present study and found that 8 weeks of CUS application reduced cell proliferation in intact male Wistar rats. Conversely, Wu and Wang (2010) used 6 weeks of CUS exposure in male Sprague-Dawley rats and did not find an effect on cell proliferation. Similarly, Lee et al. (2006) used a 19 days of CUS exposure in intact male Sprague-Dawley rats and failed to see a reduction in cell proliferation. Indeed in the present study although we found that CUS reduced cell proliferation in the dentate gyrus this effect is clearly driven by the greater reduction of cell proliferation with CUS in the castrated group.

Gonadectomy alone was seen to significantly reduce the number of Ki67-labelled cells in the GCL+SGZ, a finding that lies in contrast to those of Spritzer and Galea (2007). The discrepancies between our findings could be due to time between castration and perfusion; in the present study males were gonadectomised 4 weeks prior to tissue collection while tissue was collected roughly 1 week after gonadectomy by Spritzer and Galea (2007). Thus the discrepancy in the effects of castration on cell proliferation in the hippocampus may be due to the length of hormone withdrawal, a finding consistent with female data that shows the length of ovarian hormone withdrawal influences both cell proliferation and the expression of neuroplastic proteins (Tanapat et al., 1999; Banasr et al., 2001; Green and Galea, 2008). However, it is

important to note that the present finding of a reduction in proliferation with gonadectomy is likely driven by the greater reduction in cell proliferation levels observed in the GDX-CUS group relative to all other groups. The facilitated reduction in proliferation with CUS application in GDX males group is likely due to the absence of testicular hormones. Conversely, it can be argued that the presence of enhanced levels of testosterone observed in the Sham-CUS group facilitates proliferation in the dentate gyrus, however we do not believe this to be the case as the number of Ki67-labelled cells in the Sham-CUS group was not statistically different from the Sham-noCUS group.

2.4.5 Exposure to chronic unpredictable stress and/or gonadectomy reduces cell survival within the hippocampus

Our findings show that gonadectomy and exposure to CUS produce decreased survival of new neurons within dentate gyrus, both independently and in concert. In the present study we found that gonadectomy alone reduced the survival of new neurons in the dentate gyrus, consistent with previous findings (Spritzer and Galea, 2007). We also saw that administration of CUS, regardless of testicular hormone status, reduced the number of surviving BrdU-labelled cells in the dentate gyrus. This finding is consistent with previous research showing chronic unpredictable stress (Lee et al., 2006), chronic restraint stress (Pham et al., 2003) and chronic corticosterone administration (Karishma and Herbert, 2002; Brummelte and Galea, 2010b) reduce adult neurogenesis in male rats. Comparison of the treatment groups showed that the application of CUS and gonadectomy in tandem produced a potentiated decrease in the number of surviving BrdU-labelled cells within the GCL+SGZ of the dentate gyrus over either treatment applied alone. Neurogenesis was significantly reduced in the GDX-CUS group compared to both the

Sham-CUS and GDX-noCUS treatments, suggesting stress-effects on neurogenesis are greater in absence of testicular hormones in male rats. Furthermore, the increase in serum testosterone observed in the Sham-CUS group may have facilitated survival compared the GDX-CUS group consistent with previous findings (Spritzer and Galea, 2007).Although this possible potentiation effect does not increase the number of BrdU-labelled cells to levels observed in the Sham-noCUS group.

2.4.6 Exposure to chronic unpredictable stress and gonadectomy decreases the expression of PSA-NCAM within the hippocampus

Administration of CUS reduced the number of PSA-NCAM immunoreactive cells within the GCL+SGZ of the dentate gyrus. These findings are consistent with previous research showing exposure to chronic stress or administration of corticosterone reduces the expression of PSA-NCAM within the hippocampus of intact male rats (Nacher et al., 2004a; Conboy et al., 2009). Intriguingly, another study showed that chronic restraint stress biphasically modulates PSA-NCAM expression within the hippocampus, where 3 weeks of chronic restraint stress enhanced PSA-NCAM expression but after 6 weeks of restraint stress this enhancement was lost (Pham et al., 2003). The discrepancy with our findings of decreased expression of PSA-NCAM-labelled cells following 3 weeks of CUS, suggests that the type of stress is important in determining alterations in the expression of neuroplastic proteins. It can be further noted that our stressors were presented in an unpredictable fashion, potentially hindering the homeostatic adaptation of PSA-NCAM to the stressor. Conversely, the repeated exposure to a known stressor is likely to induce adaptive neural plasticity in the short term, an effect associated with enhanced PSA-NCAM expression, followed by decreased plasticity coinciding with habituation to the stressor.

In the present study we saw that gonadectomy alone reduced PSA-NCAM expression within the dentate gyrus, the first such finding to identify gonadal hormone modulation of PSA-NCAM expression in males. A previous study in female rats found reduced expression of PSA-NCAM within the dentate gyrus following ovariectomy (Banasr et al., 2001). Furthermore ovarian hormones modulate NCAM polysialation (Tan et al., 2009), while estradiol-induced synaptic plasticity may require PSA-NCAM (Hoyk et al., 2001). Interestingly, testosterone is aromatized to estradiol, indicating that modifications of PSA-NCAM in males by gonadectomy could be mediated by estrogens.

Finally, our current results show a significant correlation between the number of BrdU, Ki67 and PSA-NCAM immunoreactive cells. Neural progenitor cells within the adult brain express PSA-NCAM, where this neuroplastic molecule aids in the migration and integration of newly formed neurons (Burgess et al., 2008). The correlation between the number of PSA-NCAM labelled cells and enhanced proliferation may represent an increased number of cells available to express PSA-NCAM rather than an independent enhancement of polysialation. However, at least one study has shown a dissociation between the number of PSA-NCAM labelled cells and the rate of proliferation within the dentate gyrus, where PSA-NCAM expression was enhanced in the absence of alterations to proliferation or survival (Van der Borght et al., 2005).

2.4.7 Conclusions

The results of this study indicate that testicular hormones provide resilience to the development of depressive behaviours and alterations in neurogenesis and neuroplasticity in the face of unpredictable chronic unpredictable stress. Gonadectomized males exposed to three weeks of

CUS showed depressive-like behaviour, attenuated weight gain and reductions in both cell proliferation and survival as well as decreased expression of the neuroplastic protein PSA-NCAM. Thus hypogonadal males developed a more severe depressive phenotype, both behaviourally and in terms of neuroplasticity when exposed to chronic stressors compared to eugonadal males. Given the clinical literature signifying lower rates of depression in men versus women, these results demonstrate a potential advantage of male gonadal hormones, such as testosterone, in maintaining homeostatic control of stress response and enhancing neurogenesis within the limbic system. Therefore testicular hormones may confer resilience to males, reducing the likelihood of developing affective disorders including major depression. Additional research is needed to further elucidate the neurobiological mechanisms at play and further investigate the efficacy hormone replacement therapy as a treatment for depression.

3. TESTOSTERONE HAS ANTIDEPRESSANT-LIKE EFFICACY AND FACILITATES IMIPRAMINE-INDUCED NEUROPLASTICITY IN MALE RATS EXPOSED TO CHRONIC UNPREDICTABLE STRESS.[2]

3.1 Introduction

Hypogonadal males are more likely to develop depression, and conversely, depressed males are more likely to be hypogonadal (Hintikka et al., 2009; Westley et al., 2015). Testosterone supplementation has antidepressant action, as meta-analyses have shown improved mood scores in hypogonadal men following testosterone treatment (Zarrouf et al., 2009; Amanatkar et al., 2014). Testosterone supplementation also improves the response of patients to monoaminergic antidepressant drugs (Seidman and Rabkin, 1998), although evidence for this effect is limited (Orengo et al., 2005; Seidman et al., 2005; Pope et al., 2010). Interestingly, a sex difference is present in patient responsiveness to antidepressant treatment: women have a better outcome on mood scores with selective serotonin reuptake inhibitor (SSRI) treatment, whereas men have a better outcome on mood scores when treated by tricyclic antidepressants (TCAs), such as imipramine (Kornstein et al., 2000; Baca et al., 2004). These findings collectively indicate that the gonadal hormonal milieu and/or biological sex could impact the efficacy of antidepressant drugs, depending on the class of drug.

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[2]A version of this chapter has been resubmitted for publication on November 15, 2015. Wainwright SR, Workman JL, Tehrani A, Hamson DK, Chow C, Lieblich SE, Galea LAM (resubmitted November 15, 2015) Testosterone has antidepressant efficacy in a chronic unpredictable stress model of depression, independent of changes to hippocampal neuroplasticity, in castrated adult male rats .

Stress is a major preceding factor in the development of depression (Kendler et al., 1999), where dysregulation and hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis are associated with the pathogenesis of depression (Stokes, 1995; Stetler and Miller, 2011). Contrary to this, antidepressant treatment normalizes HPA function in depressed patients (Schule, 2007) in a manner coincident with an improvement in mood scores (Ising et al., 2007). The hippocampus plays an essential role in the feedback regulation of the HPA axis (Jacobson and Sapolsky, 1991), and altered hippocampal morphology often coincides with increased HPA activity (Swaab et al., 2005). Depressed patients show reduced hippocampal volume, as evidenced by a meta-analysis (McKinnon et al., 2009) and reduced neuron density in the hippocampus (Boldrini et al., 2013a). Antidepressant treatment in depressed patients, on the other hand, is associated with increased hippocampal volume, which may in part be mediated by enhanced hippocampal neurogenesis; although this effect is dependent on the age and sex of the patient (Boldrini et al., 2009; Lucassen et al., 2010; Epp et al., 2013). Neurogenesis in the hippocampus may play a key role in re-establishing basal HPA tone following stress (Snyder et al., 2011), and facilitates the normalisation of HPA feedback by antidepressant treatment (Khemissi et al., 2014). Thus, hippocampal neurogenesis is intimately tied to depression, HPA dysregulation, and perhaps specific aspects of antidepressant efficacy (Santarelli et al., 2003; David et al., 2009).

There are numerous animal models of depression, but the chronic unpredictable stress (CUS) model of depression produces the best combination of face, construct, and predictive validity for observing the pathogenesis of depression and assessing the efficacy of antidepressant agents (Willner, 2005; Hill et al., 2012). The CUS model produces numerous endophenotypes reflective of depression, including reductions in neurogenesis and neuroplasticity, that are reversed by antidepressant treatment (Vollmayr et al., 2007). Using multiple behavioural,

endocrine, and neurobiological measures following treatment with antidepressant agents, the CUS model also provides insight into potential antidepressant-like effects and the mechanisms that underlie their action (Papp et al., 1996; Willner, 2005). To our knowledge no study has fully examined antidepressant-like effects of testosterone on anhedonia, behavioural despair, and novelty-induced hypophagia within a CUS model of depression in a single study. Nor has a study fully assessed testosterone-mediated alterations to hippocampal neurogenesis and neuroplasticity within a model of depression. This study aims to fill these gaps in the literature.

We have previously modelled the effects of hypogonadism on the development of depressive-like phenotypes in male rats, showing gonadectomised males develop more pronounced behavioural and neurobiological depressive-like phenotypes than intact males when exposed to CUS (Wainwright et al., 2011). Treatment with testosterone produces antidepressant-like effects in non-stressed, intact male and female rodents (Frye and Walf, 2009), rodent models of depression using isolation stress (Carrier and Kabbaj, 2012a), and rodent models of male hypogonadism (Carrier and Kabbaj, 2012a, b); however testosterone shows no antidepressant-like behavioural effects in ovariectomized females highlighting a key sex-difference in the absence of normal gonadal tone (Carrier and Kabbaj, 2012a). Though a previous study has assessed the antidepressant-like effects of testosterone in a social-isolation model of depression, measures of behavioural despair were not used, only isolated rats were behaviourally tested, and some measures of hippocampal neurogenesis including cell survival or cell differentiation were not included (Carrier and Kabbaj, 2012a). The present study provides a thorough assessment of antidepressant efficacy in a CUS model of depression, utilizing behavioural measures that assess different mechanisms of antidepressant action (monoaminergic, neurogenic) compared to non-stress controls. Moreover, the present study assesses alterations in HPA tone, multiple aspects of

neurogenesis, and the expression of PSA-NCAM, to provide a full picture of antidepressant action.

Gonadectomy in males reduces hippocampal neurogenesis and neuroplasticity, a phenotype that resembles the effects of CUS exposure; in fact gonadectomy exacerbates the effects of stress (Spritzer and Galea, 2007; Wainwright et al., 2011; Carrier and Kabbaj, 2012b). Depressed patients show reduced hippocampal neurogenesis (Boldrini et al., 2009; Boldrini et al., 2012b), and the upregulation of neurogenesis is thought to play an integral role in antidepressant efficacy (Wainwright and Galea, 2013). Similarly, the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), which is involved in multiple forms of hippocampal neuroplasticity (Burgess et al., 2008; Rutishauser, 2008), is reduced by gonadectomy, stress, and in depressed patients; conversely PSA-NCAM expression is enhanced by antidepressant treatment (Varea et al., 2007b; Varea et al., 2007a; Wainwright et al., 2011; Guirado et al., 2012; Wainwright et al., (Submitted)). Though neurogenesis appears to play a role in antidepressant efficacy, there is strong evidence that other forms of neuroplasticity play a broader role in the action of antidepressant drugs; including evidence from our lab showing that the expression of PSA-NCAM is required for antidepressant efficacy (Bessa et al., 2009; Wainwright et al., (Submitted)).

Antidepressant drugs increase hippocampal neurogenesis via enhanced cell proliferation, with no independent effect on cell survival (Malberg et al., 2000). In contrast, chronic testosterone treatment increases hippocampal neurogenesis via an androgen receptor-dependent enhancement of cell survival, with no independent effect on cell proliferation (Spritzer and Galea, 2007; Hamson et al., 2013). Indeed, the antidepressant-like effects of testosterone do not coincide with any changes in cell proliferation in non-stressed or socially-isolated rats (Carrier

and Kabbaj, 2012a, b). Although a single study has also shown no significant enhancement of cell survival corresponding to the antidepressant-like effects of testosterone, the study was not conducted within a model of depression (Carrier and Kabbaj, 2012b). Still, it is reasonable to postulate that testosterone treatment may produce some antidepressant-like effects that coincide with an enhancement of hippocampal neurogenesis, or neuroplasticity via PSA-NCAM. To date, no study has thoroughly explored the effects of testosterone treatment in gonadectomised males in a model of depression with a specific focus on hippocampal neurogenesis and neuroplasticity.

The current study assesses the antidepressant-like efficacy of testosterone, alone and in combination with the tricyclic antidepressant imipramine, in gonadectomised males using a CUS model of depression. We examined whether testosterone alone, and in combination with imipramine, would reverse depressive-like behavioural, endocrine, and neural phenotypes in the CUS model of depression. We assessed several components of neurogenesis (proliferation, survival, and differentiation) as well as the expression of PSA-NCAM to investigate the influence of testosterone treatment on hippocampal neuroplasticity and any coincident antidepressant-like effects. We hypothesized that testosterone would show antidepressant-like effects, alone and in conjunction with imipramine treatment, in a manner that coincides with increased hippocampal neuroplasticity.

3.2 Methods

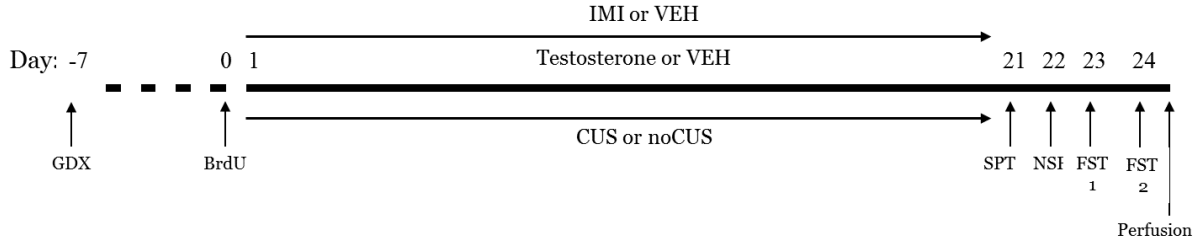
3.2.1 Subjects

Eighty male Sprague-Dawley rats were obtained from the Animal Care Center at the University of British Columbia. All rats weighed between 275-300g (approximately 60-65 days old) upon

arrival. Rats were pair-housed in standard cages with cedar bedding, paper towels, and a polyvinylchloride tube in a temperature-controlled room (21±1ºC) with a 12:12 h light/dark cycle (lights on at 0700 h). Food (Purina rat chow) and water were provided *ad libitum*, except during periods of deprivation associated with the CUS protocol.

All rats received bilateral castration, allowed one week for recovery then randomly assigned into treatment groups according to whether they received daily injections of testosterone (T) or oil, and/or treatment with the antidepressant imipramine (IMI) or vehicle (VEH), and/or received chronic unpredictable stress (CUS) or no application of stressors (noCUS). Thus there were 8 groups (n=10/group): Oil+Sal+noCUS; T+Sal+noCUS; Oil+IMI+noCUS; T+IMI+noCUS; Oil+Sal+CUS; T+Sal+CUS; Oil+IMI+CUS; T+IMI+CUS. All testing and procedures were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of British Columbia. All efforts were made to reduce the number of animals used and to minimize their suffering.

Figure 3.1: Experimental timeline of procedures and behavioural measures.



3.2.2 Surgery

Surgery was conducted one week after rats arrived in the colony using aseptic procedures. Rats weighed 300-350g on the day of surgeries. Under isoflurane anesthesia (5% in oxygen during induction, 3% in oxygen during maintenance), each rat was bilaterally castrated with both testes extracted through a small incision made at the posterior tip of the scrotum and were ligated with a monofilament suture. Immediately after surgery Flamazine cream (1% silver sulfadiazine) was applied to the incision site and each rat was given an s.c. injection of Ketoprofen (5 mg/kg body mass) as an analgesic every 24h for the three days following surgery. Rats were singly-housed following surgery and one week was allowed for full recovery from surgery, rats were pair-housed with their previous cage mate prior to further experimentation.

3.2.3 BrdU administration

On the day prior to commencement of CUS or noCUS treatment all rats were given one i.p. injection of the thymidine analog BrdU (5-Bromo-2-deoxyuridine; 200 mg/kg) 8 h apart to label dividing cells and their progeny (see Figure 3.1 for timeline). The timing of BrdU injection relative to CUS was chosen to examine the effects of CUS on neurogenesis independent of the ability of CUS to alter cell proliferation (Malberg et al., 2000). BrdU (Sigma-Aldrich, St. Louis, MO, USA) was prepared prior to injection by dissolving 20 mg/mL in warm 0.9% saline buffered with 1 N NaOH/mL saline to a physiological pH.

3.2.4 Chronic unpredictable stress

Following recovery from the castrations rats were divided into two further groups and were either subjected to 3 weeks of CUS or were left undisturbed except for weekly cage changing

(noCUS). The noCUS rats were housed in a separate colony room from rats receiving CUS in order to eliminate any potential confounds of proximity to stressed conspecifics (Sharp et al., 2002). The CUS paradigm was adapted from that previously published by our laboratory (Wainwright et al. 2011). The stress protocol consisted of the pseudo-random presentation of two stressors/day spaced at least two hours apart (See Table 3.1 for description of stressors).

Table 3.1: Stressors applied pseudo-randomly over 3-week CUS protocol.

Stressor	Description
Restraint	Animals restrained in transparent Plexiglas tubes for 1 h and returned to original cage afterward.
White Noise	Animals exposed to white noise (80 dB) for 2 h.
Stroboscopic light	Stroboscopic light (~1 flash/s) for 1 h.
Wet bedding	2 h with wet bedding (700 ml water in 350 g cage bedding). Animals placed into clean dry cage afterward.
Platform	Animals placed on 20 × 20 cm transparent Plexiglas platforms<comma> mounted on 90 cm high posts<comma> for 5 min. Animals returned to original cage afterward.
Water deprivation	18 h of water deprivation immediately followed by 1 h exposure to an empty bottle.
Food Deprivation	18 h of food deprivation immediately followed by 1 h of restricted access to food (5 pellets).
Social isolation	Animals singly housed for 18 h in small cages with a thin layer of bedding. Animals returned to original cage afterward.
Cage Tilt	Home cages tilted to a 30° angle for 2 h. without water bottles.
Soiled cage	Animals placed in a cage with soiled bedding from other animals for 1 h. Animals placed in clean cage afterward.
Tail Bleed	Blood taken from tail vein, animals then placed in a new cage.

3.2.5 Drug treatment

Rats were subdivided into drug treatment groups and underwent daily treatment with either testosterone propionate (1mg s.c.; [10mg/ml] in sesame oil) or sesame oil (0.1ml s.c.). Rats were then further subdivided and received treatment with either imipramine (10mg/kg i.p.; [10mg/ml] in 0.9% saline) or vehicle (0.9% saline i.p.). The doses chosen are consistent with the literature (Spritzer and Galea, 2007; Bessa et al., 2009; Mateus-Pinheiro et al., 2013). Both the administration of T or Oil, and IMI or VEH was concurrent with the application of the stress protocol (See Figure 3.1).

3.2.6 Sucrose preference

Preference for a 1% sucrose solution (in tap water) versus tap water was assessed each week against a baseline measurement taken prior to the commencement of the stress protocol for all rats. For testing each rat was moved to a single cage and food and water deprived for 4h prior to the presentation of a two-bottle choice (1% sucrose vs. tap water). The left-right orientation of the bottles was alternated each week to ensure side preference would not influence the results. Testing lasted 1 h and commenced at the beginning of the lights-off period (1900 h). Bottles were weighed prior to testing and immediately after to quantify the amount of liquid consumed. After testing all rats were moved back with their previous cage mate. Sucrose preference was determined by taking the proportion of sucrose solution consumed over the total amount of liquid consumed over the testing period (sucrose solution/(sucrose solution + tap water)).

3.2.7 Novelty suppressed feeding test

Prior to the novelty suppressed feeding (NSF) test, rats were food deprived for 18 h immediately following the final sucrose preference test (following procedure of (Santarelli et al., 2003; Warner-Schmidt and Duman, 2007)). The rat was placed in a novel environment (a 50 x 50 x 50cm enclosure) with a single food pellet placed in the middle of the enclosure. The latency to start feeding (during the 10 min test) was used as a measure for anxiety-like behavior and neurogenesis-dependent behavioral efficacy of antidepressant treatment (Santarelli et al., 2003; David et al., 2009). Following testing rats were returned to a cage for an hour with free access to food. During this time food consumption was measured as a control for potential appetite differences, after which rats were returned to their home cage.

3.2.8 Forced swim test

To assess depressive-like behaviour in all rats, the forced swim test (FST) was used on experimental days 23 and 24 following the NSF (Fig. 1), as previously described (Galea et al., 2001; Brummelte et al. 2006). The forced swim test is a widely recognized measure of behavioural despair in rodents (Porsolt et al., 1977a). Scoring of several active (swimming, diving, climbing, etc.) and passive (immobility, maintenance movements etc.) behaviours is done with the proportion of each determined; where administration of antidepressant drugs elevates the proportion of active behaviours (Porsolt et al., 1977a; Willner, 1984), while elevated expression of passive behaviours is representative of a "depressive" behavioural phenotype (Galea et al., 2001).

Briefly, the apparatus consisted of a vertical cylindrical glass container (45 × 28 cm) filled to a depth of 30 cm with tap water at 23 ± 0.5°C. Testing in the forced swim test was

conducted over two sessions; Session 1 took place 24 h after the administration of the NSF test and lasted 10 min, while session 2 commenced 24 h later with rats being placed in the cylinder for 5 min. Both test sessions were videotaped but only session 2 was scored and by an observer blind to conditions (Papp and Wieronska, 2000). The behaviours scored in the FST were: (1) swimming - movement of forelimbs and/or hind limbs in a paddling fashion which displaces the rat; (2) climbing - quick movements of the forelimbs such that the front paws break the surface of the water; (3) immobility - floating with the absence of any movement outside of those solely to keep their head above water. In addition, the number of fecal boli excreted by each rat was counted and recorded during each session. The latency to immobility was simply timed from the beginning of testing until a rat performed the first bout of immobility behaviour.

3.2.9 Blood collection and hormone assays

Serum levels of corticosterone were assayed for all rats. Blood was collected from the tail vein on days 10 and 21. Each sample was taken under basal conditions. All blood samples were stored overnight at 4ºC following collection. Samples were centrifuged at 10,000 rpm for 15 min, and serum was decanted and stored at -20ºC until assayed. Serum corticosterone samples were assayed in duplicate using a modified protocol from a commercially available ^{125}I radioimmunoassay kits for rat corticosterone (CORT; MP Biomedicals, Orangeburg, NY). The CORT antibody cross-reacts 100% with CORT, and shows minor cross-reactivity with deoxycorticosterone (0.34%), testosterone (0.1%), and cortisol (0.10%), but does not cross-react with progestins or estrogens (< 0.01%). The standard curve ED50 for CORT was 135.2 ng⁄ml, and the lower limit of detection for the assay was 7.7 ng⁄ml. The average intra-assay coefficient of variation for the corticosterone assay was below 10%.

3.2.10 Tissue collection

Rats were anesthetized with a lethal dose of sodium pentobarbital (Euthanyl; Bimeda-MTC, Cambridge, ON) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; Sigma Aldrich, ON, Canada) on experimental day 24 immediately following the second session of the FST. Brains were extracted and post-fixed with 4% paraformaldehyde (PFA) at 4ºC for 24 h. Brains were then cryoprotected with 30% sucrose in 0.1 M PB and stored at 4ºC until slicing. A sliding microtome was used to section the brains into 10 series of coronal tissue slices at a thickness of 40 µm. Tissue was collected throughout the entire rostral-caudal extent of the hippocampus and stored in antifreeze solution (0.05 M TBS, 30% ethylene glycol, and 20% glycerol) at -20ºC until immunohistochemical processing.

3.2.11 Immunohistochemistry

3.2.11.1 BrdU

Tissue sections were incubated at room temperature in 0.6% H_2O_2 for 30 min followed by three rinses in 0.1 M PBS prior to a 30-min incubation at 37˚C in 2 N hydrochloric acid. The tissue was then rinsed in 0.1 M borate buffer for 10 min (pH 8.5) followed by three more rinses in 0.1 M PBS. The tissue sections were then transferred to a primary antibody solution containing 1:200 mouse anti-BrdU (Roche, Indianapolis, IN), 3% normal horse serum, and 0.1% Triton X in 0.1 M PBS for 24 h at 4˚C on a shaker. The tissue was rinsed three times in 0.1 M PBS and was then transferred to a secondary antibody solution containing a 1:500 dilution of horse anti-mouse in 0.1 M PBS. The tissue was incubated in the secondary solution for 4 h at room temperature. Following three rinses in 0.1 M PBS, an ABC kit (Vector, Burlingame, CA) was used and

prepared according to the kit instructions. BrdU immunoreactive (ir) cells were visualized by incubating tissue in nickel-enhanced diaminobenzidine (DAB). Finally, the sections were mounted on glass slides and coverslipped with Permount.

3.2.11.2 Ki67

Tissue sections were first incubated at room temperature in 0.6% H2O2 for 30 min and was then rinsed three times for 10 min each in 0.1 M PBS. The sections were then transferred to a primary antibody solution containing a 1:500 Rabbit anti-Ki67 monoclonal antibody (Vector, Burlingame, CA), 3% Normal goat Serum, and 0.3% Triton-X in 0.1 M PBS. Tissue was incubated in the primary solution for 24 h at room temperature on a shaker and was then rinsed three times for 10 min per rinse in 0.1 M PBS. The tissue was then incubated for 16 h at room temperature in a secondary antibody solution containing a 1:500 dilution of goat anti-rabbit (Vector, Burlingame, CA). After rinsing the tissue three times in 0.1 M PBS, the tissue was next incubated in an ABC solution (Vector, Burlingame, CA) as per the kit instructions for 2 h. The tissue was rinsed in 0.1 M PBS and then visualized with nickel-enhanced DAB. The sections were then mounted on glass slides and were coverslipped with Permount.

3.2.11.3 BrdU/NeuN

Double labeling for BrdU and NeuN was performed to assess phenotype of the BrdU-ir cells. A series of brain tissue was incubated at 4°C for 24 h in mouse anti-NeuN (Millipore, Temecula, CA) diluted 1:250 in 0.1 M PBS containing 0.3% triton-X. The tissue was then rinsed three times in 0.1 M PBS and was then incubated for 16 h at 4°C in donkey anti-mouse Alexa 488 (Invitrogen, Eugene, OR) diluted 1:500 in 0.1 M PBS. The tissue was then rinsed three times in

0.1 M PBS and was then fixed in 4% PFA for 10 min and was then rinsed twice in 0.9% NaCl for 10 min each. DNA was denatured by heating sections to 37˚C for 30 min in 2N hydrochloric acid. BrdU labeling was then carried out by incubating the tissue for 24 h at 4°C in a 1:500 dilution of Rat anti-BrdU (AbD Serotech, Oxford, UK) in 0.1 M PBS containing 0.3% Triton-X. Following three rinses in 0.1 M PBS the tissue was then transferred to a solution containing a 1:500 dilution of Donkey anti-rat Cy3 (Jackson ImmunoResearch, Westgrove, PA) for 16 h at 4°C. Finally, the tissue was rinsed three times in 0.1 M PBS and the sections were then mounted on glass slides and coverslipped with PVA-DABCO.

3.2.11.4 PSA-NCAM

Tissue sections were incubated at room temperature in 0.3% H_2O_2 for 20 min followed by three rinses in 0.1M TBS. Sections were then transferred to a primary antibody solution containing 1:1000 mouse anti-PSA-NCAM (Millipore, Temecula, CA), 3% normal horse serum, 0.3% Triton-X in 0.1M TBS and incubated at 4˚C for 48h. The tissue was rinsed five times in 0.1M TBS at room temperature and was then transferred to a secondary antibody solution containing a 1:250 dilution of horse anti-mouse (Vector, Burlingame, CA) in 0.1M TBS and then incubated in the secondary solution for 24 h at 4˚C. Following five rinses in 0.1M TBS an ABC kit (Vector) was used and prepared according to kit instructions. PSA-NCAM-ir cells were visualized with nickel-enhanced DAB, and were then mounted on glass slides and coverslipped with Permount.

3.2.11.5 Cell counting

All counting was conducted by an experimenter blind to the experimental conditions. BrdU, Ki67, and PSA-NCAM imm unoreactive (ir) cells were counted in every 10th section throughout the entire granule cell layer (GCL), including the subgranular zone (SGZ), as well as the hilus in the case of BrdU-ir cells. Counting was performed using an Olympus light microscope under a 100x oil immersion objective lens. Cell counts were aggregated and multiplied by 10 to obtain an estimate of the total number of immunoreactive cells, as previously described (Kronenberg et al., 2003; Eadie et al., 2005; Epp et al., 2010). We also counted cells separately in the dorsal and ventral aspects of the hippocampus due to different functional differences that have been observed along the dorsoventral axis of the hippocampus, including a particular role for the ventral aspect in stress, anxiety, and depression (Fanselow and Dong, 2010). The percentage of BrdU/NeuN double-labelled cells was obtained by selecting 25 BrdU-ir cells per brain arbitrarily from at least five sections per brain and determining what percentage of these cells also expressed NeuN. Fluorescent imaging was performed on an Olympus epifluorescent microscope under 400x magnification. Area measurements of the GCL and hilus were calculated separately using the software program ImageJ (NIH). The volume of each region was estimated using Cavalieri's principle (Gundersen and Jensen, 1987). The density of immunoreactivity for each marker was determined by dividing total cell counts by the calculated areas for each of the respective regions (GCL+SGZ and hilus) as well as both the dorsal and ventral aspects of the hippocampus.

3.2.12 Data analyses

Sucrose preference, latency to feed in the NSF test, and behaviours in the FST, including the latency to the first bout of immobility were analyzed using ANOVA with stress (noCUS, CUS), hormone (oil, testosterone) and drug (vehicle, IMI) as the between-subjects factors and with FST behaviour (immobility, swimming, climbing) as within-subjects factors. Serum corticosterone levels were analysed using an ANOVA with stress (noCUS, CUS), hormone (oil, testosterone) and drug (saline, IMI) as between-subjects factors. The volume of the dentate gyrus, the density of BrdU-ir cells was analyzed with a repeated-measures ANOVA with area (GCL+SGZ, hilus) and region (dorsal, ventral) as within-subject factors and stress (noCUS, CUS), hormone (oil, testosterone) and drug (vehicle, IMI)) as between-subjects factors. The density of Ki67-ir and PSA-NCAM-ir cells were each analysed with a repeated-measures ANOVA with region (dorsal, ventral) as within-subjects factor and stress (noCUS, CUS), hormone (oil, testosterone) and drug (vehicle, IMI) as the between-subjects factors. All post hoc tests utilized Newman–Keuls unless otherwise specified. All analyses were conducted using Statistica (version 8). A priori we expected testosterone would show antidepressant action and any a priori comparisons were subjected to a Bonferroni correction. Significance level was 0.05.

3.3 Results

3.3.1 Exposure to CUS and imipramine both attenuated body mass gain, whereas testosterone increased body mass

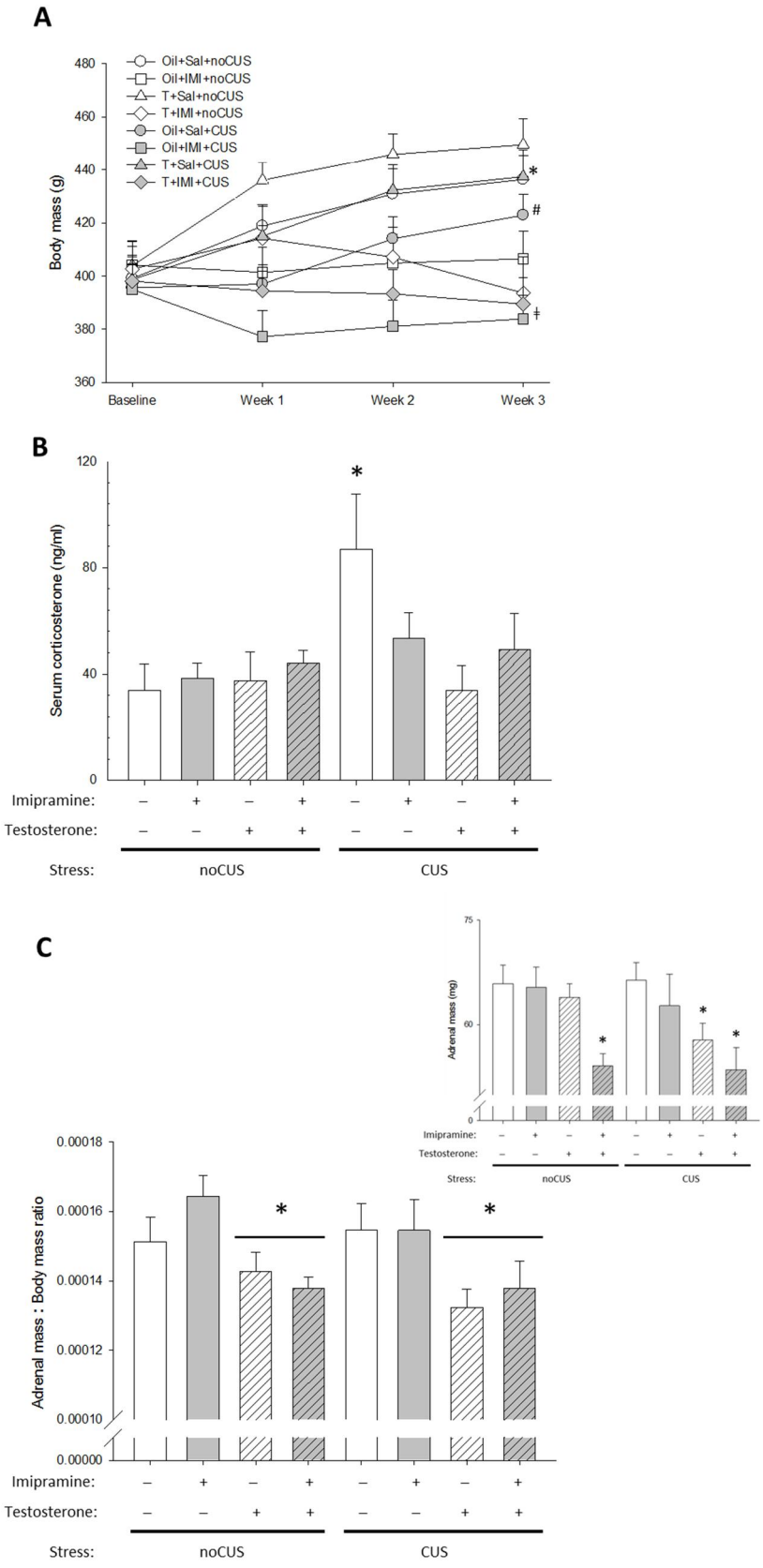
CUS treatment attenuated body mass gain in weeks 1, 2, and 3 compared to the noCUS treatment (all p's<0.0001; significant week by stress interaction ($F(3,183)=7.54$, $p<0.0001$)). Treatment

with imipramine also attenuated body mass gain in weeks 1, 2, and 3 versus saline treatment (all p's<0.0001; significant week by antidepressant interaction ($F(3,183)=59.49$, $p<0.0001$)). Conversely, testosterone treatment increased body mass gain compared to oil treatment in weeks 1 and 2 (p's<0.0001; significant week by hormone interaction ($F(3,183)=6.59$, $p=0.0003$)). There were also main effects of stress ($p<0.03$) and antidepressant ($p<0.0003$) (see Figure 3.2A).

3.3.2 Administration of testosterone reduced basal serum corticosterone levels in CUS-exposed groups. Testosterone and imipramine reduced adrenal mass

CUS exposure increased serum CORT ($p<0.04$); however, testosterone treatment decreased serum CORT in CUS exposed rats compared to those treated with oil ($p<0.03$, hormone by stress interaction; ($F(1,64)=4.12$, $p<0.05$), see Figure 3.2B. There were no further significant effects (all p's>0.12). Testosterone reduced relative adrenal mass (adrenal/body mass ratio) regardless of other treatments (main effect of hormone, $F(1,61=15.26$, $p<0.0003$); see Figure 3.2C). There were no other significant effects on relative adrenal mass (all p's>0.22). Testosterone (main effect of hormone, ($F(1,65)=14.20$, $p<0.0004$)) and imipramine (main effect of antidepressant, ($F(1,65)=4.94$, $p<0.03$)) also both reduced total adrenal mass regardless of CUS treatment (See Figure 3.2C inset). There were no other significant effects on adrenal mass (all p's>0.23).

Figure 3.2: (A) Mean (+ SEM) body mass on the commencement of chronic unpredictable stress (CUS) or noCUS exposure (Baseline), Weeks 1, 2 and 3 of experimentation. Exposure to CUS (#$p<0.001$), and imipramine (ǂ$p<0.001$) treatment significantly attenuated weight gain compared to noCUS controls. Testosterone treatment significantly increased body mass compared to CUS exposed vehicle groups (*$p<0.001$). (T = Testosterone; Sal = Saline; IMI = Imipramine; CUS = Chronic unpredictable stress) **(**B) Exposure to chronic unpredictable stress (CUS) significantly increased the mean, SEM; Day 10 and 21 pooled) concentration of serum corticosterone compared to non-stressed (noCUS) groups (*$p<0.04$). Treatment with testosterone attenuated the CUS-induced increase in serum corticosterone. (C) Treatment with testosterone significantly reduced adrenal to body mass ratio regardless of other treatment (*$p<0.001$). Inset graph shows treatment with testosterone and imipramine, alone or in tandem, decreased mean (± SEM) adrenal mass compared to chronic unpredictably stressed (CUS) or non-stressed (noCUS) controls (*$p<0.03$).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3.3.3 Imipramine reduces latency to feed in the NSF in all groups, whereas testosterone reduced latency to feed under CUS conditions only

Imipramine treatment alone, and in combination with testosterone reduced latency to feed under both CUS and noCUS conditions (all p's<0.0002), while testosterone treatment alone reduced latency to feed only under CUS conditions compared to vehicle treatment ($p<0.0003$; hormone x antidepressant x stress interaction: $F(1,61)= 7.51$, $p<0.009$; see Figure 3.3A). However, imipramine in combination with testosterone did not reduce latency to feed further than IMI or T alone under CUS conditions (p's>0.3) or than IMI under noCUS conditions ($p>0.87$). There were no treatment effects on food consumption following testing (all p's>0.15; see Table 3.2).

Table 3.2: Food consumed (mean ± SEM) following the novelty suppressed feeding test across all groups. (T = Testosterone; Sal = Saline; IMI = Imipramine; CUS = Chronic unpredictable stress).

Treatment	*N*	Food Consumed (g)
Oil+Sal+noCUS	9	6.33 ± 0.73
Oil+IMI+noCUS	9	5.78 ± 0.68
T+Sal+noCUS	9	4.67 ± 0.73
T+IMI+noCUS	8	5.88 ± 0.64
Oil+Sal+CUS	10	7.00 ± 0.70
Oil+IMI+CUS	8	6.13 ± 0.48
T+Sal+CUS	10	6.50 ± 0.65
T+IMI+CUS	10	5.70 ± 0.50

3.3.4 Testosterone enhanced the antidepressant action of imipramine to increase sucrose preference under CUS conditions

Analysis of the final sucrose preference test revealed a hormone by antidepressant interaction ($F_{1,62}=6.13$, $p=0.016$), showing that testosterone in combination with imipramine treatment increased preference compared to testosterone ($p<0.02$), or imipramine ($p<0.05$), treatment alone (see Figure 3.3B).

3.3.5 Testosterone and imipramine treatment both independently increase the latency to first immobility in the forced swim test

Overall, there were no significant effects of stress, hormone, or antidepressant treatment on the proportion of time spent swimming, struggling, or immobile during the forced swim test (all p's>0.12; see Table 3.3). However, the latency to first immobility was significantly increased by testosterone (main effect of hormone; ($F(1,63)=7.40$, $p<0.009$)) and imipramine treatment (main effect of antidepressant; ($F(1,63)=5.97$, $p<0.02$; see Figure 3.3C), regardless of CUS group. A priori comparisons show that it is only the combined treatment of testosterone and imipramine that increases latency to first immobility compared to vehicle treatment under both noCUS and CUS conditions (p's <0.01, 0.02 respectively).

Figure 3.3: (A) Imipramine treatment decreased the mean (± SEM) latency to feed in the novelty supressed feeding test, regardless of stress condition. Testosterone treatment only decreased latency to feed under the chronic unpredictable stress (CUS) condition. There was no significant additive effect of testosterone and imipramine treatment (*$p<0.03$). (B) Mean (±SEM) sucrose preference (percentage of baseline sucrose preference) on the final day of testing (Day 21) compared to baseline preference was significantly greater in the testosterone plus imipramine group exposed to chronic unpredictable stress (CUS), but not in non-stressed (noCUS) groups (*$p<0.02$). (C) The combination of testosterone and imipramine treatment significantly increased the mean (± SEM) latency to the first bout of immobility in the forced swim test, under both chronic unpredictable stress (CUS) and non-stress (noCUS) conditions (*$p<0.02$).

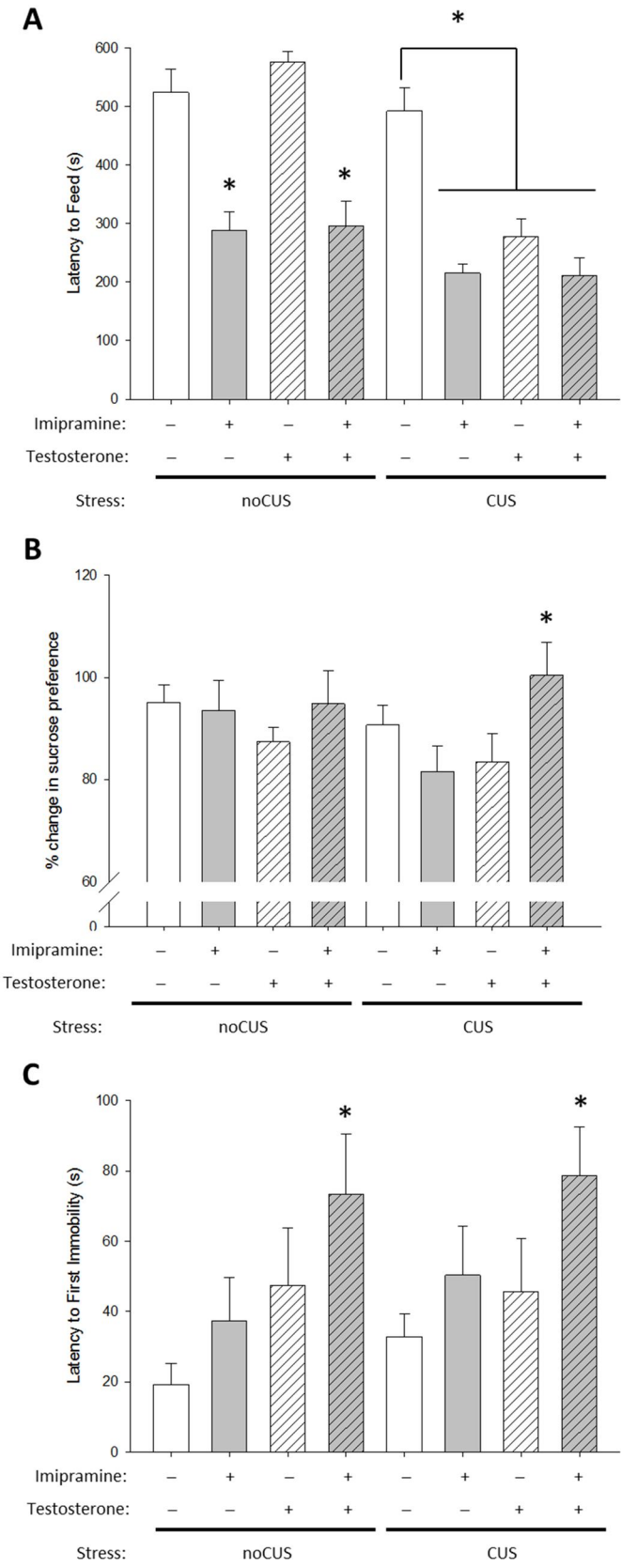
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Table 3.3: Mean (± SEM) percentage of time spent showing individual behaviours in the forced swim test across all groups. There were no significant effects observed between treatment groups. (T = Testosterone; Sal = Saline; IMI = Imipramine; CUS = Chronic unpredictable stress).

		Immobility		Swim		Struggle	
Treatment	**N**	Mean (%)	SEM	Mean (%)	SEM	Mean (%)	SEM
Oil+Sal+noCUS	8	37.77	5.41	46.83	5.87	15.40	2.62
Oil+IMI+noCUS	9	40.00	3.24	33.70	5.80	26.29	3.76
T+Sal+noCUS	9	31.80	6.09	43.40	5.89	24.80	4.01
T+IMI+noCUS	8	37.86	5.65	33.96	3.84	28.18	5.32
Oil+Sal+CUS	10	27.59	3.08	46.29	5.84	26.11	4.24
Oil+IMI+CUS	9	37.22	4.90	34.92	5.74	27.86	5.15
T+Sal+CUS	10	35.09	4.80	41.56	5.28	23.36	2.95
T+IMI+CUS	10	27.64	5.52	46.98	6.77	25.38	3.55

3.3.6 There were no significant treatment effects on the volume of the dentate gyrus

Analysis of dentate gyrus volumes revealed a main effect of area ($F(1,67)=702.49$, $p<0.0001$), as expected, where the hilus has a greater volume than the GCL. There were no other significant main effects or interactions (all p's>0.11; see Table 3.4).

Table 3.4: Granule cell layer (GCL) and Hilus volumes (mean ± SEM) of the dentate gyrus for each treatment group. There were no significant effects observed between treatment groups. (T = Testosterone; Sal = Saline; IMI = Imipramine; CUS = Chronic unpredictable stress).

		GCL		Hilus	
Treatment	**N**	Mean (mm^3)	SEM	Mean (mm^3)	SEM
Oil+Sal+noCUS	9	2.67	0.14	5.07	0.29
Oil+IMI+noCUS	10	2.67	0.14	5.42	0.31
T+Sal+noCUS	10	2.39	0.12	4.54	0.32
T+IMI+noCUS	8	2.68	0.13	4.96	0.38
Oil+Sal+CUS	9	2.59	0.12	5.01	0.39
Oil+IMI+CUS	10	2.59	0.15	5.05	0.30
T+Sal+CUS	9	2.93	0.16	5.26	0.30
T+IMI+CUS	10	2.66	0.17	4.81	0.30

3.3.7 Imipramine increased PSA-NCAM expression in the ventral dentate gyrus

Imipramine increased the number of PSA-NCAM-expressing cells in the ventral dentate gyrus ($p<0.005$) but not dorsal dentate gyrus ($p=0.48$), regardless of CUS condition (region by antidepressant: $F(1,65)=5.87$, $p<0.02$; see Figure 3.4A). There was also a main effect of region ($p<0.0001$) but no other significant effects (all p's>0.22). Intriguingly, under CUS conditions testosterone and imipramine treatment together increased the number of PSA-NCAM expressing

cells in the ventral dentate gyrus compared to the vehicle treatment ($p<0.024$), but not imipramine ($p=0.47$) or testosterone alone ($p=0.89$).

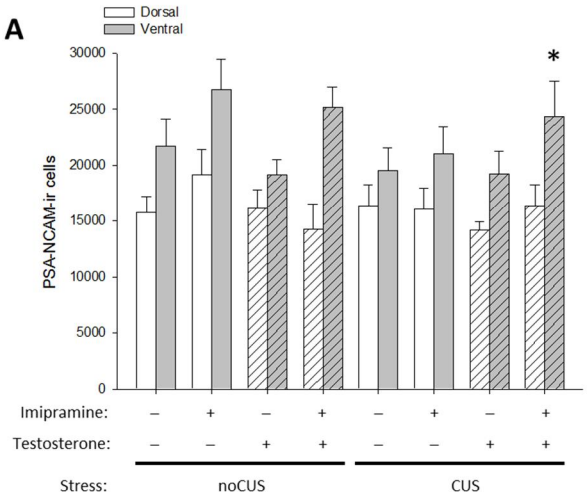
3.3.8 Imipramine increased cell proliferation in the ventral GCL

Imipramine increased the number of Ki67-ir cells in the ventral region of the GCL ($p<0.003$), but not the dorsal region ($p>0.41$; area by antidepressant interaction ($F(1,66)=4.53$, $p<0.04$)), regardless of CUS group (see Figure 3.4B). There was also a main effect of region on number of Ki67-ir cells ($F(1,66)=238.54$, $p<0.0001$) but no other significant effects (all p's>0.09; see Figure 4B inset).

3.3.9 CUS decreased the number of BrdU-ir cells in the ventral GCL, whereas imipramine increased the number of BrdU-ir cells in the dorsal GCL and decreased the number of BrdU-ir cells in the ventral GCL only under noCUS conditions

Exposure to CUS decreased the number of BrdU-ir cells in the ventral GCL ($p<0.0004$), with no significant effect in the dorsal GCL ($p=0.21$; region by stress interaction: $F(1,60)=14.4$, $p<0.0003$; see Figure 3.4C). Under noCUS conditions, imipramine increased the number of BrdU-ir cells in the dorsal GCL ($p<0.0002$) but decreased the number of BrdU-ir cells in the ventral GCL ($p<0.0002$; region by area by antidepressant by stress interaction: ($F(1,60)=21.19$, $p<0.00001$) but there were no significant effects under CUS conditions of imipramine. As expected, there were main effects of region (dorsal vs. ventral; $p<0.0001$) and area (GCL vs. hilus; $p<0.0001$) but no other significant main effects.

Figure 3.4: (A) Testosterone plus imipramine treatment significantly increased the mean (± SEM) number of PSA-NCAM-ir cells in the ventral area of the dentate gyrus under chronic unpredictable stress conditions (CUS), but not the non-stressed (noCUS) condition (*$p<0.03$). (B) Imipramine significantly increased the mean (± SEM) number of Ki67-ir cells in ventral region of the dentate gyrus, compared to the saline treatment groups. Graph indicates groups collapsed across chronic unpredictable stress (CUS) condition and testosterone treatment while inset graph indicates all groups (*$p<0.04$). (C) Exposure to CUS decreased the number of BrdU-ir cells in the ventral dentate gyrus, regardless of group and figure indicates groups collapsed across testosterone treatment (ǂ$p<0.001$). Imipramine treatment increased the mean (± SEM) number of BrdU-ir cells in the dorsal region of granule cell layer of the dentate gyrus (*$p<0.0002$), and decreased the number of BrdU-ir cells in the ventral region (#$p<0.0003$) compared to saline treatment under non-stressed (noCUS) conditions. Inset graph indicates all groups (CUS = chronic unpredictable stress).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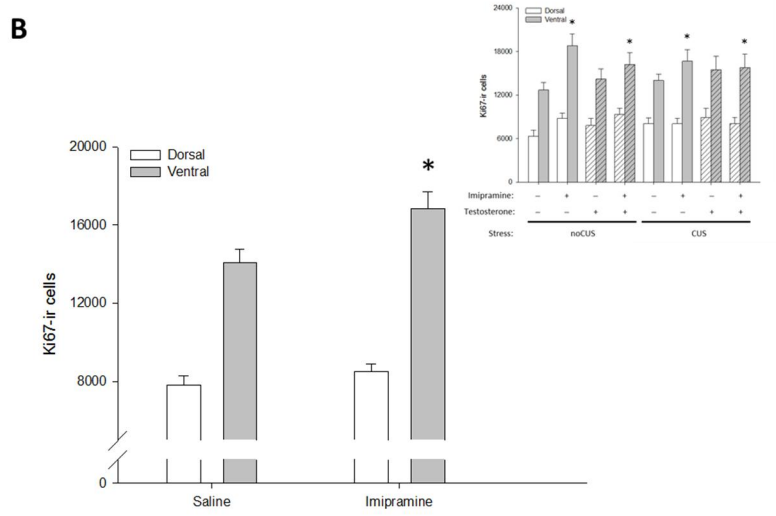
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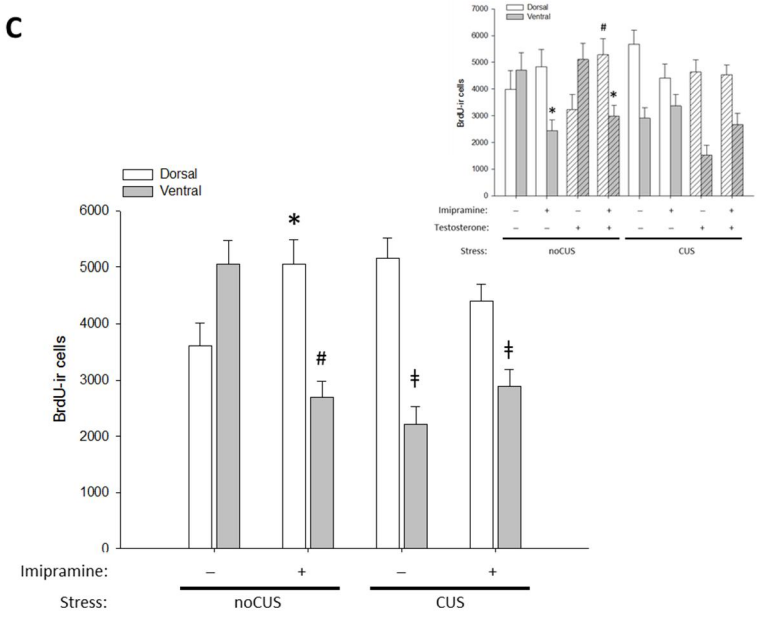
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noCUS
CUS

3.3.10 The dorsal GCL had a greater proportion of BrdU/NeuN-ir cells than the ventral region of the GCL. There were no significant treatment effects on the proportion of BrdU-ir cells differentiating into neurons

There was a greater proportion of BrdU/NeuN-ir cells in the dorsal area of the dentate gyrus compared to the ventral region (main effect of region ($F(1,55)=4.03$, $p<0.05$; see Table 3.5). There were no significant effects of CUS, imipramine, or testosterone treatment alone, or in any combination (all p's>0.11).

Table 3.5: Mean (± SEM) percentage of BrdU/NeuN-ir cells separated by region of hippocampus. There were no significant effects observed between treatment groups. (T = Testosterone; Sal = Saline; IMI = Imipramine; CUS = Chronic unpredictable stress).

		Dorsal		Ventral	
Treatment	**N**	Mean (%)	SEM	Mean (%)	SEM
Oil+Sal+noCUS	7	81.50	3.76	77.56	1.95
Oil+IMI+noCUS	8	83.65	3.06	80.37	2.23
T+Sal+noCUS	6	88.44	3.75	83.12	1.26
T+IMI+noCUS	8	86.94	2.02	82.37	3.09
Oil+Sal+CUS	9	88.60	2.91	82.91	3.53
Oil+IMI+CUS	8	83.17	4.80	82.33	3.26
T+Sal+CUS	7	86.36	3.26	86.26	1.41
T+IMI+CUS	10	79.59	2.31	80.98	3.10

3.4 Discussion

Our findings demonstrate that testosterone effectively attenuates some depressive-like behaviours, as well as physiological and endocrine depressive-like endophenotypes within a chronic unpredictable stress model of depression in adult castrated males. Testosterone shows antidepressant-like efficacy both independently, and as an adjunct treatment to imipramine, in a behaviour-, and state-dependent manner. As expected, imipramine alone showed antidepressant effects in FST and NSF test; however, we did not observe an independent effect of imipramine treatment on anhedonia in the sucrose preference test. Imipramine treatment also increased cell proliferation and PSA-NCAM expression in the ventral dentate gyrus, while increasing the survival of BrdU-ir cells in the dorsal dentate gyrus. Testosterone treatment alone did not produce an enhancement of hippocampal neurogenesis, though testosterone treatment along with imipramine increased expression of PSA-NCAM in the ventral dentate gyrus. These findings show that 3-weeks of testosterone treatment alone did not significantly upregulate hippocampal neurogenesis or PSA-NCAM expression, however testosterone was effective in reducing endocrine and behavioural depressive-like endophenotypes, while facilitating an antidepressant-induced enhancement of PSA-NCAM after CUS. These findings show that testosterone exerts antidepressant-like effects in a multifaceted manner and can act to improve efficacy of the tricyclic imipramine in castrated male rodents in a CUS model of depression.

3.4.1 Testosterone treatment attenuated the effects of CUS exposure on body mass, adrenal mass and basal corticosterone levels

The rats exposed to CUS showed reduced body mass compared to the noCUS exposed rats across all three weeks of treatment, consistent with our previous study using the 3-week CUS

paradigm in gonadectomised males (Wainwright et al., 2011); a finding reminiscent of weight change often seen in depressed patients (Weissenburger et al., 1986; Stunkard et al., 1990) Testosterone treatment inhibited the effect of CUS to attenuate gaining body mass, such that body mass was statistically equivalent to the noCUS controls, and increased body mass compared with vehicle-treated rats during the first two weeks of treatment. As expected imipramine, a known anorexic agent, significantly reduced body mass (Blavet and DeFeudis, 1982), whereas testosterone treatment assuaged this effect; although testosterone + imipramine did not enhance body mass back to control levels.

Application of the CUS paradigm increased basal serum corticosterone levels, again replicating our previous findings (Wainwright et al., 2011) and consistent with findings in depressed patients (Stetler and Miller, 2011). This finding strengthens the face validity for our CUS paradigm, as this depressive-like endocrine phenotype resembles the HPA hyperactivity observed depressed patients (Holsboer and Barden, 1996; Stetler and Miller, 2011). Both testosterone, and imipramine, independently reduced the CUS-induced increase in basal serum corticosterone concentration, to levels observed in the noCUS treatment groups. Treatment with testosterone or imipramine similarly decreased the mass of the adrenal glands, again suggesting decreased HPA reactivity (Raone et al., 2007). Interestingly, normal HPA negative feedback inhibition is predictive of successful remission with antidepressant treatment in depressed men, but not in women (Ising et al., 2007; Binder et al., 2009). It is well established that testosterone enhances negative feedback of the HPA axis at multiple levels in an AR-dependent manner (Viau, 2002), our findings therefore support a role for this mechanism in the antidepressant-like endocrine effects of testosterone treatment.

3.4.2 Testosterone and imipramine alone decreased latency to feed in the novelty-suppressed feeding test but testosterone only under CUS conditions

Our findings show that imipramine functioned as expected, reducing the latency to feed in the NSF test in both CUS and noCUS exposed rats (Santarelli et al., 2003; Bessa et al., 2009; Carrier and Kabbaj, 2012a). Testosterone treatment also reduced the latency to feed in the NSF test, a result that is in accordance with the findings of Carrier and Kabbaj (2012a). However, we only observed antidepressant-like effects of testosterone in rats exposed to CUS. Indeed, Carrier and Kabbaj (2012a) only assessed the antidepressant and anxiolytic efficacy of testosterone treatment in rats exposed to isolation stress, and not in control rats. Thus, this study extends beyond previous findings through the use of a CUS model of depression and demonstrating the state-dependent antidepressant-like effects of testosterone, wherein latency to feed is only reduced by testosterone under stress conditions. Aligning with the findings of Carrier and Kabbaj (2012a), we did not see an additive effect of testosterone plus imipramine over testosterone or imipramine treatment alone, under CUS conditions. Given the doses of imipramine used in the present study (10mg/kg), and in Carrier and Kabbaj (2012a) (20mg/kg), any additive effects of testosterone treatment may have been masked by latency to feed being reduced to a floor level by imipramine alone. The results of this study therefore suggest the neural and hormonal milieu against which testosterone replacement is acting may be critically important to the suppression of novelty-induced hypophagia.

The anxiolytic effects of testosterone must also be considered for the effect of testosterone in the NSF test. Previous studies have shown acute and chronic testosterone treatment produces anxiolytic effects in an androgen receptor (AR)-dependent manner (Edinger and Frye, 2006; Hodosy et al., 2012). Other studies have however, pointed to the aromatization

of testosterone to estradiol or products of 5α-reductases, such as 3α-diol, with activity at the estrogen receptor (ER) β, as the mediators of anxiolytic- and antidepressant-like effects of testosterone (Frye and Edinger, 2004; Frye et al., 2008; Carrier et al., 2015). Given that testosterone can be converted into either dihydrotestosterone (DHT) or estradiol, it may work to exert its effects via ER or AR. Length of treatment, and latency between treatment and testing may play a role in the expression of the behavioural effects of testosterone, with one study demonstrating rapid non-genomic anxiolytic effects of estradiol treatment in gonadectomised males (Filova et al., 2015). It therefore appears that there are time, receptor, and state-dependent anxiolytic-like and antidepressant-like effects of testosterone.

3.4.3 Testosterone treatment produced antidepressant-like effects in the forced swim test

We found that both testosterone and imipramine independently increased the latency to immobility in the FST, regardless of CUS treatment, an effect representative of antidepressant-like action of testosterone and consistent with studies using intact, gonadectomised, and stressed male rodents (Bernardi et al., 1989; Buddenberg et al., 2009; Frye and Walf, 2009; Carrier and Kabbaj, 2012a). However, in the present study our findings revealed no significant difference in the total time spent demonstrating any behaviour (immobility, swimming, struggling), regardless of stress, testosterone, or antidepressant treatment. Our findings are somewhat inconsistent with previous studies showing antidepressant-like efficacy of testosterone in the forced swim test that includes reducing the total time spent immobile in addition to reducing the latency to immobility (Carrier and Kabbaj, 2012b). It is important to note that there are some major difference between the studies assessing the antidepressant-like effects of testosterone in the forced swim test; including the use of intact versus gonadectomised animals; using osmotic mini-pumps versus

subcutaneous injection for the administration of testosterone; the length of testosterone treatment; and the use of non-stressed versus chronically stressed test subjects (Buddenberg et al., 2009; Frye and Walf, 2009; Carrier and Kabbaj, 2012a; Carrier et al., 2015). Dose-dependent effects of testosterone administration in the forced swim test have been shown, where 2mg/kg and 4mg/kg acute doses show greater efficacy than the 1mg/kg dose as used in the present study (Buddenberg et al., 2009). Given these experimental differences, and given the dose-dependent nature of antidepressant-like effects of testosterone, it was pertinent to assess the latency to immobility, as it is effective in elucidating antidepressant-like action (Mezadri et al., 2011), and in fact shows increased sensitivity over the measurement of the duration of behaviours (Castagne et al., 2009).

Testosterone plays a key role in mediating the antidepressant effects of tricyclic antidepressants, but not SSRIs, in the FST (Martinez-Mota and Fernandez-Guasti, 2004). However, our findings do not imply a specific enhancement of noradrenergic or serotonergic neurotransmission as struggling or swimming behaviour respectively (Detke et al., 1995). Testosterone, at least in the lower dose used in this study, may therefore specifically reduce the latency to immobility in the CUS model. Hippocampal glucocorticoid receptors play a role in mediating immobility behaviour, as reduced GR expression or activation coincides with decreased immobility (Korte et al., 1996; Bachmann et al., 2005; Solomon et al., 2014). Treatment with DHT reduces GR expression in the hippocampus, suggesting an AR-dependent mechanism (Kerr et al., 1996). However, estradiol treatment also modulates GR expression in a manner that coincided with anxiolytic-like and antidepressant-like effects (Carrier et al., 2015). It is therefore possible that the antidepressant-like effects of testosterone in the FST observed in the present study may be mediated through both AR- or ER-dependent mechanisms.

3.4.4 Testosterone enhanced the antidepressant-like effects of imipramine on sucrose preference

We found that only the combination of testosterone and imipramine treatment increased sucrose preference over the non-treated CUS controls on the last day of treatment. Surprisingly, we did not observe an independent effect of imipramine (Muscat et al., 1990; Papp et al., 1994; Bekris et al., 2005) or testosterone to increase sucrose preference, as have been previously reported (Carrier and Kabbaj, 2012b, a; Carrier et al., 2015). The discrepancy with previous studies is likely due to methodological differences including the type of stress exposure, the dose of testosterone, the length of administration, the route of administration, and the length of sucrose presentation (Muscat et al., 1990; Sampson et al., 1991; Muscat et al., 1992; Carrier and Kabbaj, 2012b, a; Carrier et al., 2015), as described above. Although, our findings do support previous studies that have shown testosterone enhances the antidepressant efficacy of imipramine to increase sucrose preference in castrated males (Carrier and Kabbaj, 2012a). Imipramine enhances sucrose preference via a dopamine-dependent mechanism mediated through the D_1 and/or D_2 dopamine receptors (Muscat et al., 1990). Indeed the efficacy of other antidepressants, including SSRIs, in the sucrose preference test is also dependent upon activation of the D_1 or D_2 receptors (Sampson et al., 1991; Muscat et al., 1992). However, the only other study to assess the efficacy of imipramine in the sucrose preference test in castrated males used a dose of 20mg/kg delivered via osmotic minipump. Therefore the dose of imipramine may need to be higher in castrated males, compared to intact or testosterone supplemented males to increase sucrose preference. Indeed, the rewarding properties of testosterone are mediated via activation of mesolimbic D_1 and D_2 receptors, a mechanism that aligns with the antidepressant effects of imipramine (Schroeder and Packard, 2000). Testosterone may therefore facilitate the

antidepressant action of imipramine on sucrose preference through the enhancement of D_1 and/or D_2 activation.

Previous studies have shown that estradiol treatment increases sucrose preference in a manner similar to that of testosterone treatment, whereas treatment with the aromatase inhibitor fadrozole, inhibits the hedonic effects of testosterone, indicating a ER-dependent mechanism of testosterone (Carrier et al., 2015). Indeed, previous studies have shown that a reduction in mesolimbic dopamine can be ameliorated by treatment with either testosterone or estradiol, but not DHT (Mitchell and Stewart, 1989). The 5α-reduced testosterone metabolite 3α-diol is also a strong ERβ agonist (Kuiper et al., 1997), and appears to mediate some of the hedonic properties of testosterone (Frye, 2007). It therefore appears that the effect of testosterone in producing antidepressant-like action in combination with imipramine observed in the present study is mediated through ER; however, it is outside the scope of the current study to suggest a definitive mechanism.

3.4.5 Testosterone treatment did not significantly alter measures of hippocampal neurogenesis or neuroplasticity alone. Imipramine treatment increased neurogenesis

Exposure to CUS reduced neurogenesis in the ventral dentate gyrus, but not the dorsal dentate gyrus. This finding is consistent with previous studies showing a region-specific reduction in neurogenesis in response to chronic unpredictable stress (Jayatissa et al., 2006). In the present study imipramine enhanced cell proliferation in a robust manner within the ventral GCL, the region largely associated with stress responsivity and affect (Fanselow and Dong, 2010). This is consistent with some previous studies that have shown antidepressant upregulation of neurogenesis in the ventral GCL (Banasr et al., 2006; Jayatissa et al., 2006). Testosterone

treatment had no significant effect on cell proliferation, replicating previous findings from our laboratory and others (Spritzer and Galea, 2007; Carrier and Kabbaj, 2012b, a). Similarly, we observed no significant treatment effects on the differentiation of newly generated neurons in the dentate gyrus, corresponding with previous findings showing no effect of imipramine (Mateus-Pinheiro et al., 2013), or testosterone treatment on neural differentiation (Spritzer and Galea, 2007; Hamson et al., 2013).

As expected, imipramine increased survival of BrdU-ir cells, although we only observed a significant increase in the dorsal dentate gyrus, suggesting a region-specific effect that, to our knowledge, has not been previously described. Few studies have assessed the effect of antidepressant on cell survival along the dorsoventral axis, showing equivalent increases in both regions and none of these studies have assessed the effects of imipramine (O'Leary and Cryan, 2014). We observed no significant effect of testosterone treatment on cell survival, a finding consistent with a previous report (Carrier and Kabbaj, 2012b) but inconsistent with reports from our own laboratory (Spritzer and Galea, 2007; Hamson et al., 2013). Previous studies using 30 days of testosterone treatment observed increased neurogenesis (via increased BrdU-ir cell survival) in an androgen receptor-dependent manner (Spritzer and Galea, 2007; Hamson et al., 2013). Therefore the 21-days of testosterone treatment used in the present study, and by Carrier et al., (2012b), appears to be an insufficient length of treatment to enhance neurogenesis in gonadectomised males, as further evidenced in the literature (Galea et al., 2013). Given these findings, the antidepressant-like effects of testosterone observed in the present study do not appear to coincide with an enhancement of hippocampal neurogenesis.

3.4.6 Imipramine enhanced PSA-NCAM expression, an effect facilitated by co-treatment with testosterone

The expression of PSA-NCAM was significantly increased in the ventral GCL by the combination of imipramine and testosterone, a finding consistent with previous reports of chronic antidepressant treatment in intact males, including previous findings from our laboratory (Guirado et al., 2012; Wainwright et al., (Submitted)). The expression of PSA-NCAM is required for the efficacy of SSRI treatment (Wainwright et al., (Submitted)), and the upregulation of NCAM expression in the hippocampus is associated with the antidepressant efficacy of imipramine in the FST and sucrose preference test within a CUS model of depression (Bessa et al., 2009). The additive effects of testosterone and imipramine treatment in measures of anhedonia, and behavioural despair (two measures independent of alterations to neurogenesis) coinciding with enhanced hippocampal PSA-NCAM observed in the present study, may therefore indicate a mechanism of antidepressant action in these measures.

There is a paucity of research regarding the influence of testosterone on the expression of PSA-NCAM in the hippocampus, or elsewhere in the brain. Previous research showed that gonadectomy reduced the expression of PSA-NCAM in the dentate gyrus, an effect exacerbated by CUS exposure (Wainwright et al., 2011). In the present study testosterone treatment alone showed no significant effect on the expression of PSA-NCAM in the dentate gyrus, suggesting testosterone may not play a role in modulating PSA-NCAM-dependent neuroplasticity or that the dose of testosterone was insufficient to significantly upregulate expression. However, previous research in female rodents has shown estradiol treatment enhances PSA-NCAM expression in the dentate gyrus (Banasr et al., 2001), although there are no studies assessing the effects of estradiol on PSA-NCAM expression in males to our knowledge. It is possible that an ER-

dependent mechanism is present in males, but the levels of estradiol resulting from the aromatisation of testosterone may have been insufficient to produce a significant upregulation of PSA-NCAM in the present study.

3.4.7 Conclusion

Here we show that testosterone treatment produces state-dependent antidepressant-like behavioural and endocrine effects in gonadectomised male rats. However, treatment with testosterone alone did not alter measures of hippocampal neurogenesis or PSA-NCAM expression in the dentate gyrus. These findings show testosterone exerts antidepressant effects across multiple modalities, with some aspects facilitating the antidepressant actions of imipramine, effectively modelling clinical data. Indeed, testosterone supplementation in hypogonadal men shows antidepressant-like efficacy, facilitates antidepressant action in some treatment-resistant patients. Better understanding the physiological and neurobiological means through which testosterone exerts antidepressant like effects will improve patient outcome and may facilitate the development of novel, more efficacious, drugs.

4. ENZYMATIC DEPLETION OF THE POLYSIALIC ACID MOIETY ASSOCIATED WITH THE NEURAL CELL ADHESION MOLECULE INHIBITS ANTIDEPRESSANT EFFICACY.[3]

4.1 Introduction

Multiple classes of antidepressants exist, however no pharmacological antidepressant has remission rates above 50%, and many patients relapse due to loss of efficacy (Byrne and Rothschild, 1998). The increasing global burden of depression places a priority on further delineating the mechanisms underlying antidepressant action, and subsequently the exploration of new targets for intervention (Ferrari et al., 2013). Several theories, and lines of evidence, exist regarding the pathogenesis of depression and the modes of antidepressant efficacy, including the modulation of neurotransmission, the upregulation of neurogenesis and neurotrophic factors, normalizing hypothalamic-pituitary-adrenal (HPA) reactivity, and the reduction of neuroinflammation (Holsboer, 2000; Dantzer et al., 2008; Wainwright and Galea, 2013). Therefore, an ideal molecular target for pharmaceutical intervention would function at the confluence of these theories.

Chronic stress and the dysregulation of the HPA axis are linked to depression (Anisman and Zacharko, 1982). The hippocampus contains the greatest concentration of glucocorticoid receptors, regulates the HPA axis, and is vulnerable to chronic stress and depression

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[3]A version of this chapter has been accepted for publication. Wainwright SR, Barha CK, Hamson DK, Epp JR, Chow C, Lieblich SE, Rutishauser U, Galea LAM (in press, October 27, 2015) Ezymatic depletion of the polysialylated form of the neural cell adhesion molecule inhibits antidepressant efficacy.

(Sapolsky, 1986). Depressed patients show decreases in hippocampal neurogenesis, neuropil density, and total volume (Stockmeier et al., 2004; Boldrini et al., 2009); conversely, chronic antidepressant treatment is associated with the stabilization of the HPA axis (Ising et al., 2007), increased hippocampal neurogenesis and greater hippocampal volume (Boldrini et al., 2009; Epp et al., 2013). Capitalizing on this association between stress and depression, we used a chronic unpredictable stress (CUS) protocol to induce a depressive-like phenotype in rats which has good face, construct and predictive validity (Willner, 2005; Vollmayr et al., 2007; Wainwright and Galea, 2013).

The neural cell adhesion molecule (NCAM) is a protein expressed on the surface of most neural cells and functions to mediate cell-cell interactions (Rutishauser, 2008). NCAM is subject to post-translational modifications, the most prevalent of which is glycosylation by polysialic acid (PSA), forming polysialylated neural cell adhesion molecule (PSA-NCAM). PSA attenuates NCAM-NCAM interactions and interferes with cell adhesion (Rutishauser, 2008). PSA-NCAM is required for proper cell migration, neurite outgrowth, and for activity-induced synaptic plasticity, including LTP; although the ablation of PSA-NCAM does not affect basal synaptic transmission (Muller et al., 1996; Burgess et al., 2008; Rutishauser, 2008; McCall et al., 2013). PSA-NCAM interacts with neuroreceptors (Brezun and Daszuta, 2000; Black et al., 2009; Grzegorzewska et al., 2010), the glucocorticoid receptor (Rodriguez et al., 1998), and with neurotrophic factors directly associated with antidepressant effects such as BDNF via the TrkB and p75 receptors, and FGF receptors 1 and 2 (Vutskits et al., 2001; Aonurm-Helm et al., 2008; Elsayed et al., 2012). PSA-NCAM is a member of the immunoglobulin superfamily and functions in the immune response, directly interacting with protein complexes associated with inflammation and linked to the pathogenesis of depression (Krushel et al., 1999; Koo et al.,

2010). It is important to note that the ablation of neurogenesis (Santarelli et al., 2003; Bessa et al., 2009; David et al., 2009), serotonergic neurotransmission (Lieben et al., 2006), or other proteins associated with antidepressant efficacy such as p11 (Egeland et al., 2010), does not induce a depressive-like behavioural phenotype, however the $NCAM^{-/-}$ knockout does indeed produce a depressive-like phenotype (Aonurm-Helm et al., 2008) in an animal model, suggesting a potentially unique contribution to the pathophysiology of depression, and to its treatment.

In this study we examined whether PSA-NCAM is required for the antidepressant efficacy of the selective-serotonin reuptake inhibitor fluoxetine (FLX). PSA-NCAM was selectively depleted using the intracerebroventricular (ICV) infusion of the bacteriophage-derived enzyme endoneuraminidase N (EndoN) (Rutishauser et al., 1985) prior to the administration of FLX within our CUS model of depression. We then evaluated the requirement of PSA-NCAM for antidepressant efficacy using standardized neurogenesis-dependent and -independent behavioural measures (David et al., 2009) in addition to measures of neuroplasticity within the hippocampus including synaptic density, neurogenesis (proliferation, survival, differentiation) and neural activation (using the immediate early gene cFos). Through the direct disruption of the PSA moiety we demonstrated that PSA-NCAM is required for antidepressant efficacy and that in its absence neuroplasticity is attenuated, and the behavioural efficacy of fluoxetine is completely inhibited.

4.2 Methods

4.2.1 Subjects

Sixty-six male Sprague-Dawley rats (University of British Columbia) weighing ~300g upon arrival were used. Rats were pair-housed under standard laboratory conditions (12:12 h light/dark cycle with lights on at 0700 h, at 21±1ºC, food and water provided *ad libitum*). Rats were randomly assigned to eight treatment groups according to whether they received ICV infusion of EndoN or saline, and/or received CUS or noCUS, and/or received fluoxetine (FLX) or vehicle (VEH). The 8 groups were: Sal+noCUS+VEH (n=8); Sal+noCUS+FLX (n=8); Sal+CUS+VEH (n=8); Sal+CUS+FLX (n=10); EndoN+noCUS+VEH (n=8); EndoN+noCUS+FLX (n=8); EndoN+CUS+VEH (n=8); EndoN+CUS+FLX (n=8). Sample sizes were chosen from previous studies (Santarelli et al., 2003; David et al., 2009; Wainwright et al., 2011). All procedures were carried out in accordance with the Canadian Council on Animal Care guidelines and were approved by the local Animal Care Committee.

4.2.2 Surgery

Surgeries were conducted 6-10 days after rats arrived, using aseptic procedures. Under isoflurane anesthesia each rat received either a single, unilateral ICV infusion of EndoN (1µl; 180U/µl) or Vehicle (1µl; 0.9% saline) via a Hamilton syringe (coordinates of AP: -1, ML: -1.5, DV: -3.7 from bregma). Marcaine (0.5mg), buprenophrine (0.02 mg/kg) and ketoprofen (5 mg/kg) were used as analgesics. Rats were pair-housed and given 6 days for recovery (Figure 4.1).

4.2.3 BrdU administration

One day prior to beginning CUS or noCUS treatment all rats were given two i.p. injections of 5-Bromo-2-deoxyuridine (BrdU; 200 mg/kg) 8h apart. The timing was chosen to examine the effects of CUS on neurogenesis independent of the ability of CUS to alter cell proliferation. BrdU (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving 20 mg/mL in 0.9% saline buffered with 2N NaOH.

4.2.4 Chronic unpredictable stress procedure and antidepressant treatment

All rats were subjected to 6 weeks of either CUS or noCUS. The noCUS rats were housed in a separate colony room from rats receiving CUS to eliminate any odor confounds of stressed conspecifics. The CUS protocol consisted of the pseudo-random presentation of two stressors per day (Wainwright et al., 2011). The first 3 weeks of CUS was to develop a depressive-like phenotype, while during the final 3 weeks of CUS (or noCUS) all rats underwent concurrent daily treatment with either fluoxetine (FLX; 10mg/kg; i.p.; in 0.9% saline and 10% DMSO) or vehicle and the experimenter was not blind during this process.

4.2.5 Sucrose preference test

For testing each rat was moved to a single cage, food and water deprived for 4h, then presented with two pre-weighed bottles containing a 1% sucrose solution or tap water for 1h (at 1900 h). Sucrose preference was calculated blind as: (sucrose intake/(sucrose intake + water intake)) x 100). Anhedonia was determined as the proportion of sucrose preference at week 6 against a baseline level taken prior to the commencement of CUS.

4.2.6 Novelty suppressed feeding test

Rats were food-deprived for 18h then placed in a novel environment with a food pellet in the center of the arena (50 x 50 x 50cm). The latency to start feeding (max 10min) was measured blind to assess anxiety-like behavior. Following testing, food consumption was measured (1h) as a control for appetite differences.

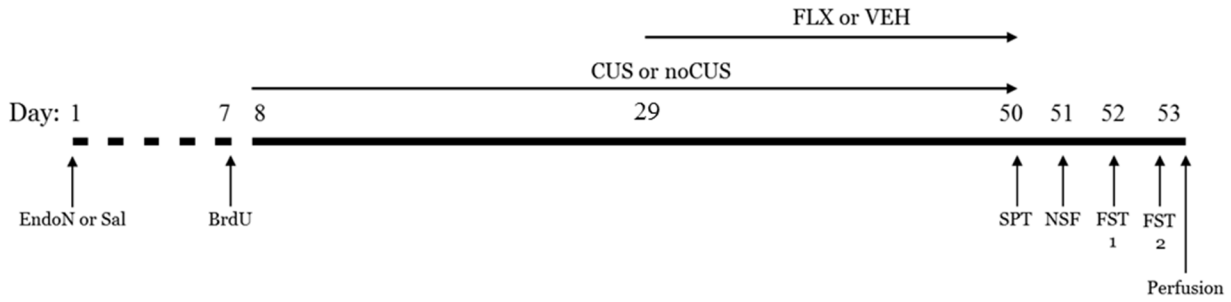
4.2.7 Forced swim test

Stress-coping behavior was assessed using the forced swim test (Wainwright et al., 2011). Briefly, rats were placed in a cylindrical glass container filled to a depth of 50 cm with water at 23±0.5°C. The forced swim test was conducted over two sessions 24h apart lasting 15min (session-1) and 5 min (session-2). Videotaped sessions were scored blind for swimming, climbing, and immobility.

4.2.8 Hormone assays

Blood was collected under basal conditions from the tail vein each week from the CUS groups, and only on weeks 1, 3, and 6 from the noCUS groups to avoid additional stress. Perfusion blood was collected for testosterone assay. Samples were stored overnight at 4ºC, centrifuged at 10,000rpm for 15min, and serum was stored at -20ºC. Samples were assayed in duplicate using a modified protocol from a commercially available ^{125}I radioimmunoassay kits for testosterone (MP Biomedicals, Costa Mesa, CA) and rat corticosterone (MP Biomedicals, Orangeburg, NY).

Figure 4.1: Experimental timeline



4.2.9 Histology

Rats were anesthetized with a lethal dose of sodium pentobarbital (Euthanyl; Bimeda-MTC, Cambridge, ON) and perfused transcardially (0.9% saline followed by 4% paraformaldehyde) 90min after the forced swim test. Brains were extracted and post-fixed with 4% paraformaldehyde for 24h, then cryoprotected with 30% sucrose and stored (4ºC). Brains were sectioned throughout the rostral-caudal extent of the hippocampus into series of 40µm coronal slices. Brain sections were stained for PSA-NCAM (mouse anti-PSA-NCAM 1:1000; Millipore, Temecula, CA); Ki67, an endogenous marker of cell proliferation (Rabbit anti-Ki67 1:500; Vector, Burlingame, CA); cFos, an immediate early gene (rabbit anti-cFos 1:1000; Santa Cruz Biotechnology, Inc.); PSD-95, an endogenous measure of synaptic density (1:1000; Abcam, Inc.); BrdU, to assess cell survival (mouse anti-BrdU 1:200; Roche, Indianapolis, IN); or double-labelled BrdU/ NeuN to measure neuron differentiation (mouse anti-NeuN 1:250; Millipore, Temecula, CA; rat anti-BrdU; AbD Serotech, Oxford, UK). An ABC kit (Vector Laboratories) was used for detection, with diaminobenzadine as the chromogen (Sigma; Oakville, ON Canada). BrdU/NeuN immunostained sections were incubated with conjugated fluorescent

secondary antibodies (BrdU-1:500 donkey anti-rat Cy3 (Jackson ImmunoResearch, Westgrove, PA); NeuN-1:500 donkey anti-mouse Alexa 488 (Invitrogen, Eugene, OR)).

BrdU, Ki67, cFos and PSA-NCAM immunoreactive (ir) cells were counted blind on an Olympus light microscope at 1000x magnification in every 10^{th} section throughout the granule cell layer (GCL), including the subgranular zone (SGZ) and hilus. Counts were aggregated and multiplied by 10 to estimate the total number of immunoreactive cells, as described(Epp et al., 2010). Counts were separated by the dorsal and ventral hippocampus, as the ventral hippocampus is specifically implicated in depression(Fanselow and Dong, 2010). The percentage of BrdU/NeuN double-labelled cells was obtained by selecting 25 BrdU-ir cells per brain arbitrarily from at least five sections per brain. Fluorescent imaging was performed on an Olympus epifluorescent microscope under 400x magnification, and verified using confocal microscopy. Area measurements of the GCL and hilus were calculated using the program ImageJ (NIH). Volume was estimated using Cavalieri's principle.

The optical density of PSD-95 expression at 40x magnification was calculated using ImageJ on photomicrographs with fixed light intensity and exposure settings acquired using cellSens software (Olympus Canada Inc., Toronto, ON). Mean grey levels were measured by pseudo-randomly placing open circles along different regions of the entire extent of the hippocampus: dentate gyrus (GCL; both supra- and infrapyramidal blades), CA1 (stratum radiatum), and CA3 (stratum lucidum). The background grey level for each section was obtained from the mean of three circles placed within the corpus callosum to control for any potential variations in staining. A threshold was then set at three-times the background mean grey level, and the mean grey level above the threshold for each region was measured, averaging the grey levels of all circles within that region, adapted from (Snyder et al., 2012).

4.2.10 Data analyses

Factorial or repeated-measures ANOVA were conducted on each behavioural, neural or endocrine measure with stress (noCUS, CUS), enzyme (Sal, EndoN) and drug (VEH, FLX) as the between-subjects factors. For repeated-measures ANOVA within-subjects factors were: forced swim test behavior (immobility, swimming, climbing), week (baseline, 3 and 6) for hormones, area (GCL+SGZ, hilus) and region (dorsal, ventral) for volume, or region (dorsal, ventral) for density of Ki67-ir, PSA-NCAM-ir, and cFos-ir cells or hippocampal field (DG, CA1, CA3) for PSD-95. Any violations of homogeneity of variance used Hyundt-Feldt corrections (cFos, BrdU) and violations in normality (CORT) were transformed. Subjects were excluded if brain tissue was damaged resulting in missing sections for histology (n=2-4), if performance was either not recorded due to equipment malfunction or deemed an outlier (greater than 2 standard deviations (n=2-4), or not enough serum for duplicates/high coefficient of variation (n=10). All post hoc tests utilized Newman–Keuls. We expected EndoN would attenuate antidepressant action and any *a priori* one-tailed comparisons were subjected to a Bonferroni correction. Significance level was 0.05.

4.3 Results

4.3.1 EndoN treatment disrupts the behavioural efficacy of fluoxetine

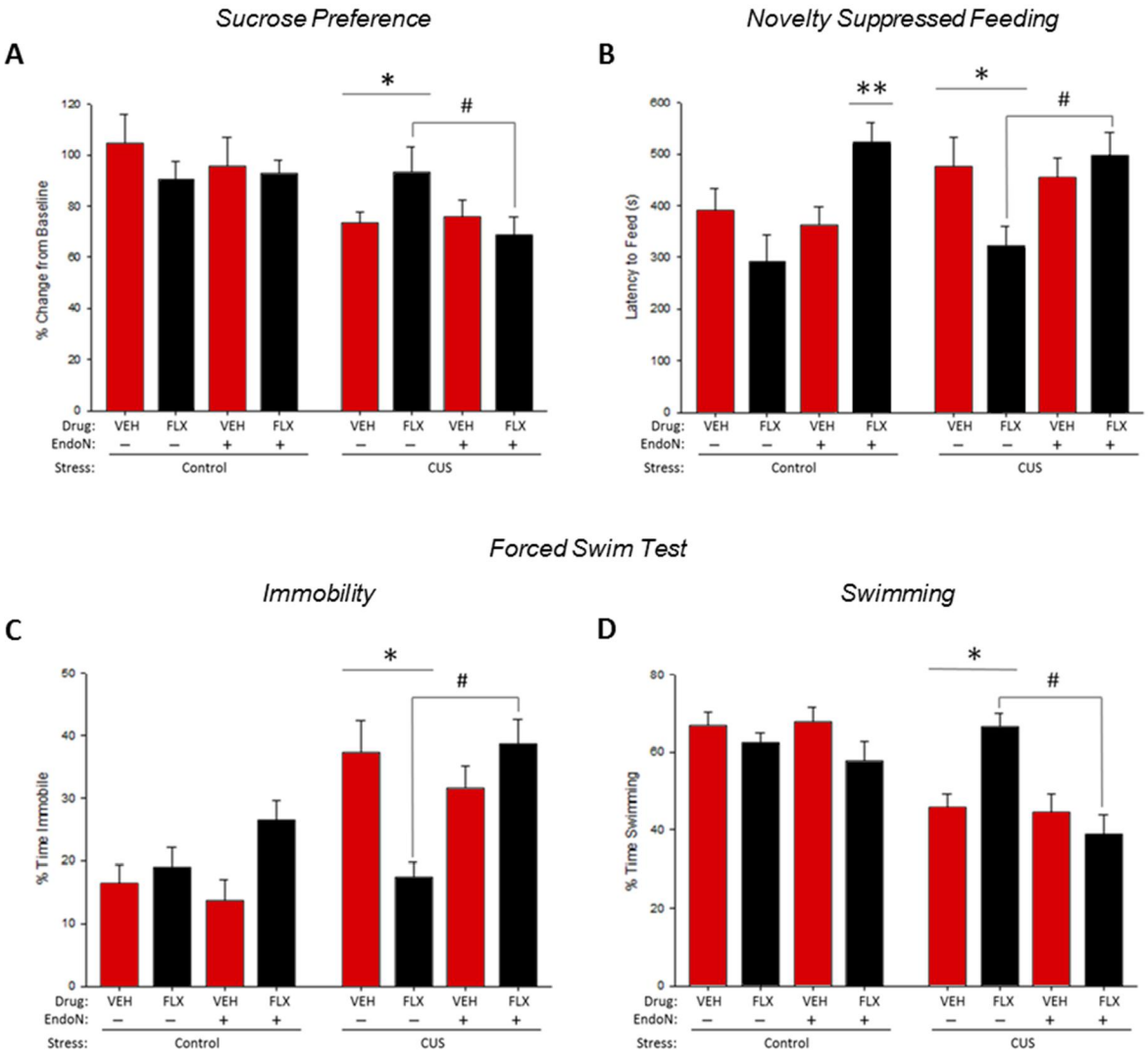
CUS treatment reduced sucrose preference (Figure 4.2A; main effect of stress ($p<0.005$), and FLX treatment increased sucrose preference in CUS rats ($p<0.05$). Interestingly, EndoN inhibited the effect of FLX treatment on sucrose preference as the EndoN-CUS-FLX group had a lower preference than the Sal-CUS-FLX group (*a priori* $p<0.04$). EndoN treatment showed no

independent effect on sucrose preference (main effect of enzyme $p>0.25$), suggesting no basal disruption based on enzyme treatment.

FLX treatment, regardless of CUS, decreased latency to feed in the novelty suppressed feeding test but only in the absence of EndoN treatment ($p's<0.03$; enzyme by drug interaction (Figure 2B, $p<0.0005$)). EndoN inhibited the effects of FLX treatment as the EndoN-CUS-FLX and EndoN-noCUS-FLX groups had a significantly greater latency to feed than their controls (Sal-CUS-FLX ($p<0.008$), and the Sal-noCUS-FLX ($p<0.0005$) group). Importantly, there were no significant differences between any of the groups (all $p's > 0.19$) in food consumption following the test indicating no appetite differences between groups.

As expected, CUS increased immobility and decreased swimming behavior while FLX treatment decreased immobility and increased swimming behavior, compared to VEH treatment (Figure 2C and 2D; all $p's<0.04$; behavior by stress by drug interaction: $p>0.002$). EndoN functioned to eliminate the effects of FLX treatment on immobility ($p<0.001$) and swimming ($p<0.0005$) compared to the saline-infused treatment groups (behavior by enzyme by drug ($p<0.001$), behavior by enzyme by stress ($p<0.05$).

Figure 4.2: Endoneuraminidase N (EndoN) inhibits antidepressant efficacy of fluoxetine (FLX) following chronic unpredictable stress (CUS)-induced behavioural changes (A) Anhedonia was assessed in the sucrose preference test. Mean ± SEM sucrose preference was reduced with CUS, FLX increased sucrose preference but this effect was inhibited by EndoN treatment (*$p<0.05$, #$p<0.04$). (B) Anxiety- and depression-like behavior was assessed in the novelty-suppressed feeding test as Mean ± SEM latency to feed in seconds. In the CUS–exposed rats FLX decreased latency to feed, while EndoN treatment inhibited the effects of FLX to reduce latency to feed (*$p<0.03$. **$p<0.008$, #$p<0.0005$). (C and D) Learned-helplessness was evaluated in the forced swim test. Mean ± SEM immobility was increased after CUS-exposure, FLX decreased percentage time spent immobile, while EndoN inhibited FLX effects (*$p<0.04$, #$p<0.001$). (D) Mean ± SEM swimming behavior was decreased after CUS, while FLX increased but treatment with Endo inhibited the effects of FLX on percent time spent swimming (*$p<0.04$. #$p<0.0005$). n= 7-9 per group.



4.3.2 EndoN treatment reduced neuroplasticity and attenuated some of FLX-induced increases in neuroplasticity

As expected, EndoN treatment significantly reduced the density of PSA-NCAM-ir cells across all groups in both the dorsal and ventral dentate gyrus (DG; Figure 4.3A, 4.3E, 4.3F; all p's <0.0005; enzyme by region interaction: $p<0.03$; enzyme by drug interaction: $p<0.05$).

Both EndoN and CUS significantly decreased the density of BrdU-ir cells in the GCL+SGZ (Figure 4.3B; $p<0.0002$), but not in the hilus ($p>0.94$; area by enzyme interaction $p<0.00001$; area x stress interaction $p<0.04$). Endo-CUS-FLX had a lower density of BrdU-ir cells than the Sal-CUS-FLX group in both the ventral and dorsal GCL (all p's <0.038). EndoN and CUS both reduced the proportion of BrdU/NeuN co-labeled cells (Figure 4.3G-K) in the GCL, with the effect of CUS most pronounced in the dorsal region (region by enzyme by stress interaction $p<0.02$). FLX treatment increased the density of Ki67-ir cells in the ventral ($p<0.04$) but not dorsal ($p>0.9$) GCL, regardless of group (Figure 4.3C; region by drug interaction: $p<0.042$).

In response to acute swim stress, CUS groups showed reduced cFos-ir expression (main effect of stress: $p<0.044$). EndoN attenuated the effect of FLX to reduce the density of cFos-ir cells in the ventral DG (Figure 4.3D; region by enzyme by drug interaction: $p<0.022$).

EndoN reduced PSD-95 expression in all three regions (Figure 4.4A-E; $p<0.018$, main effect of enzyme). FLX increased PSD-95 expression under CUS condition only, regardless of region (stress by drug interaction ($p=0.009$). EndoN treatment attenuated the enhancement of PSD-95 expression by FLX in both the CA3 ($p<0.016$) and DG ($p<0.03$) regions but not in the CA1 region ($p=0.51$) under CUS conditions.

Figure 4.3: Endoneuraminidase N (EndoN) attenuates some measures of neuroplasticity following fluoxetine (FLX) treatment and chronic unpredictable stress (CUS) (A) Mean ± SEM density of PSA-NCAM-ir cells in the granule cell layer (GCL). EndoN reduced PSA-NCAM-ir cells in the dentate gyrus regardless of CUS or FLX (*$p<0.0005$, #$p<0.0003$). (B) Mean ± SEM density of BrdU-ir cells in the GCL. EndoN and CUS reduced neurogenesis in the ventral GCL. However, FLX treatment reduced neurogenesis when given to the EndoN treated group (*$p<0.04$, #$p<0.04$). (C) Mean ± SEM density of Ki67-ir cells in the GCL. FLX treatment increased cell proliferation (Ki67-ir cells) in the ventral region compared to VEH treatment, regardless of group (*$p<0.04$). (D) Mean ± SEM density of cFos-ir cells in the GCL. Expression of cFos-ir cells was reduced in the CUS groups after acute swim stress (*$p<0.05$), while under CUS EndoN reversed the FLX-induced decrease in cFos-ir cells (#$p<0.03$). (E-I) Photomicrographs of (E) PSA-NCAM in vehicle treated and (F) EndoN treated groups with a fluorescent secondary antibody and taken with a confocal microscope for improved clarity and contrast. Photomicrographs of (G) NeuN-ir cells in the GCL, (H) BrdU-ir cells in the GCL and (I) merged BrdU/NeuN-ir cell with confocal stacks in the x-z plane (J) and in the y-z plane (K). n= 6-10 per group.

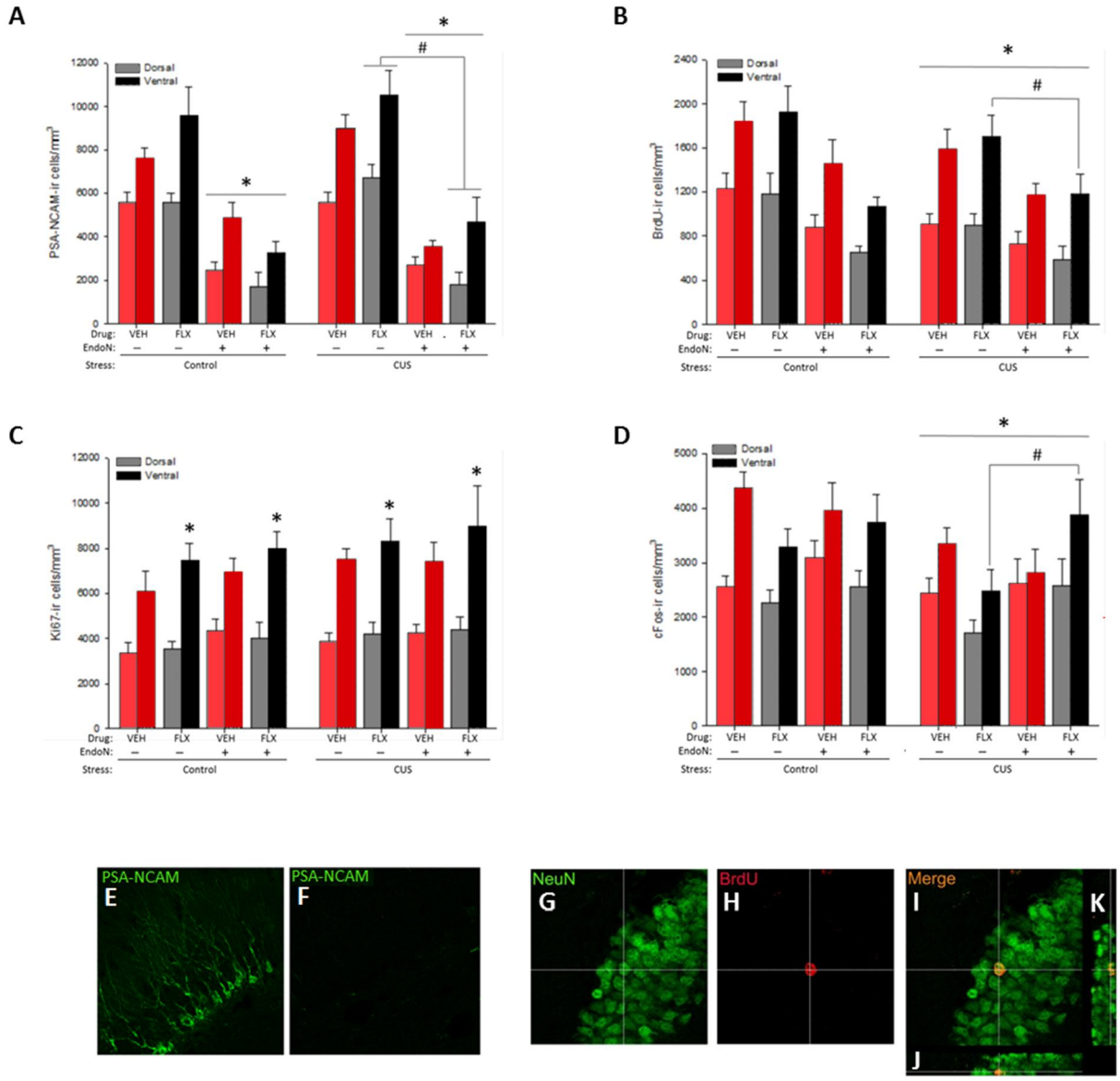
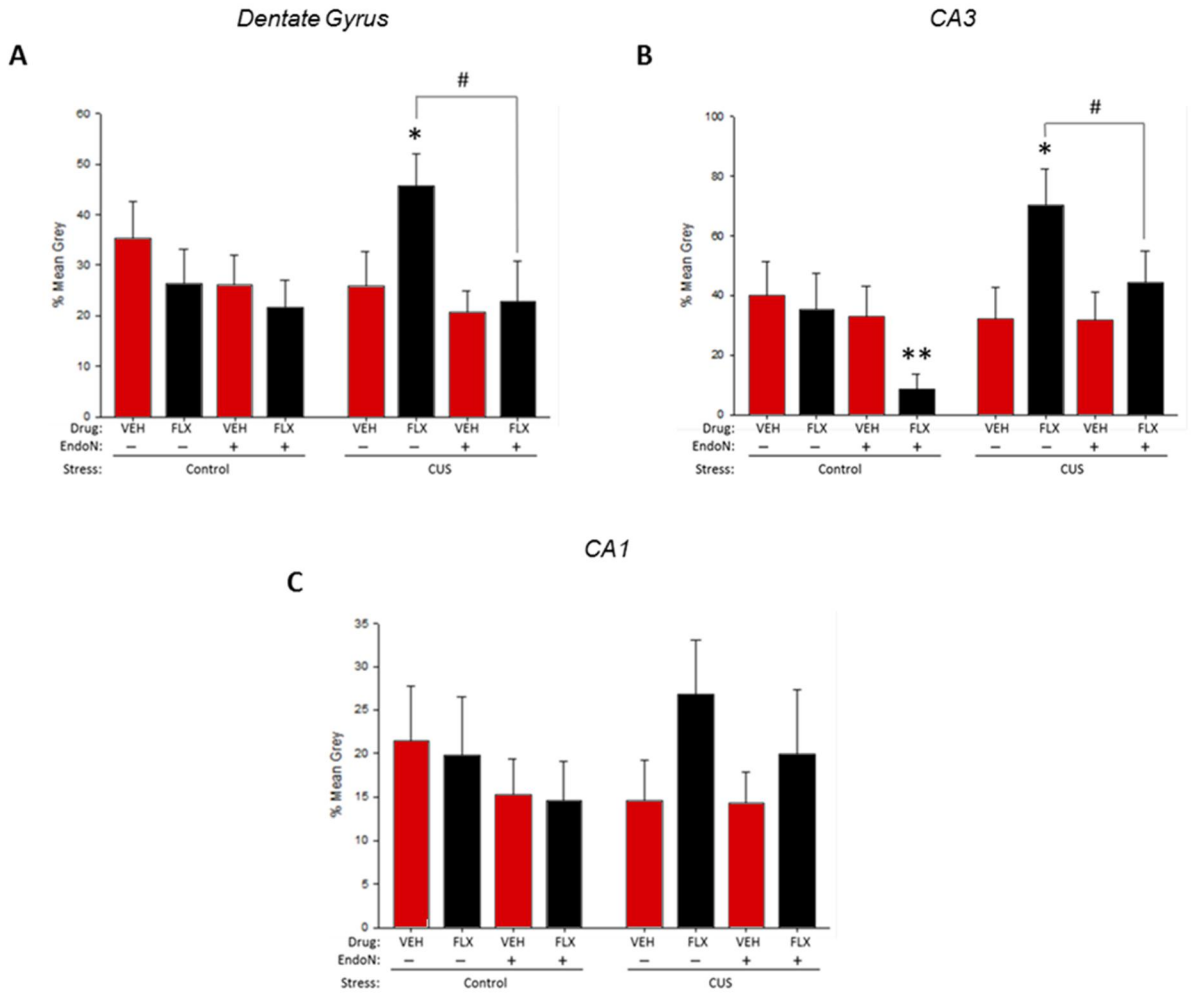

A
B
C
D
Dorsal
Ventral
PSA-NCAM-ir cells/mm3
BrdU-ir cells/mm3
Ki67-ir cells/mm3
cFos-ir cells/mm3
Drug: VEH FLX VEH FLX VEH FLX VEH FLX
EndoN: – – + + – – + +
Stress: Control CUS
*
#
PSA-NCAM
E
PSA-NCAM
F
NeuN
G
BrdU
H
Merge
I
K
J

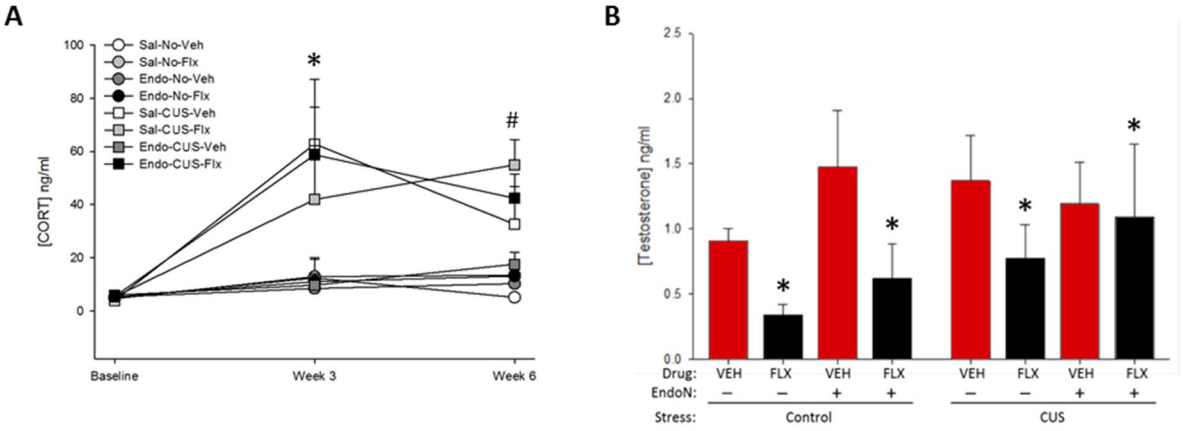
Figure 4.4: Endoneuraminidase N (EndoN) attenuates the fluoxetine (FLX) -stimulation of PSD-95 expression in the dentate gyrus and CA3, but not CA1. Mean ± SEM grey values for optical density of PSD-95 expression. (A) Optical density was increased by FLX in CUS-exposed rats, an effect inhibited by EndoN (*p<0.009, #p<0.03). (B) Optical density was increased by FLX in CUS-exposed rats, an effect inhibited by EndoN treatment in CA3. EndoN treatment also decreased optical density in control rats (*p<0.009, **p<0.05, #p<0.03). (C) No significantly treatment effects on optical density were observed in CA1. n= 7-9 per group.



4.3.3 EndoN treatment attenuated CUS-induced increase in basal corticosterone levels in the saline-treated animals. Fluoxetine decreased testosterone levels

CUS increased basal CORT levels (weeks 3 and 6) regardless of drug or enzyme treatment (Figure 5A: stress by week interaction: $p<0.0002$). However, under CUS EndoN-treated rats showed no significant increase in CORT (p's >0.27), indicating the basal CORT response was eliminated in vehicle-treated animals. FLX treatment decreased testosterone levels, regardless of group (Figure 5B $p<0.034$; main effect of drug: $F(1,47)=4.73$, $p=0.034$).

Figure 4.5: Chronic unpredictable stress (CUS) increased basal corticosterone level, and fluoxetine (FLX) reduced serum testosterone levels. (A) Mean ± SEM basal serum corticosterone level. As expected CUS exposure increased basal corticosterone; however, FLX failed to reduce basal corticosterone levels and EndoN did not significantly attenuate FLX effects (*$p<0.0002$, #$p<0.03$). (B) Mean ± SEM serum testosterone level. Regardless of group, FLX reduced testosterone levels. (*$p<0.04$). n= 6-8 per group.



4.4 Discussion

We found that PSA-NCAM is required for the behavioural antidepressant efficacy of FLX and necessary for multiple facets of FLX-induced neuroplasticity, but not the effects of FLX on endocrine measures. EndoN, which selectively cleaved PSA from NCAM, inhibited the antidepressant effects of FLX in each behavioural measure and attenuated the effects of FLX on synaptic protein expression, activation of neurons, and neurogenesis. Thus, the proper expression of PSA-NCAM is essential to the generation of antidepressant effects of FLX.

4.4.1 CUS induced a depressive-like phenotype that was reversed by FLX

Our CUS protocol was successful in developing behavioural, endocrine, somatic, and neural depression-like endophenotypes consistent with the literature (Willner, 2005; Vollmayr et al., 2007). Rats exposed to CUS showed: (1) decreased sucrose preference representative of anhedonia, (2) increased immobility in the forced swim test, indicative of increased passive stress-coping behaviour, (3) increased basal CORT levels, (4) decreased hippocampal neurogenesis (BrdU/NeuN expression), and (5) decreased synaptic density (PSD-95 expression). CUS decreased cFos expression in the dentate gyrus in response to acute stress. Our results also demonstrate a replication of the well-established antidepressant effects of chronic FLX treatment within both behavioural and neural measures. Briefly, FLX reversed CUS-induced anhedonia in the sucrose preference test, learned helplessness in the forced swim test, anxiety-like behaviour in the novelty suppressed feeding test, while increasing hippocampal neurogenesis, PSD-95 expression, and PSA-NCAM expression. Having established a viable animal model of depression encompassing numerous endophenotypes and replicating the well-defined

antidepressant effects of FLX, we were able to successfully determine the role of PSA-NCAM in antidepressant efficacy utilizing EndoN.

4.4.2 EndoN eliminated the behavioural efficacy of FLX

The infusion of EndoN prevented the FLX-mediated increase in sucrose preference in CUS exposed rats, thereby functionally disrupting antidepressant action. The antidepressant effects of FLX on sucrose preference are mediated by the D_2-receptor (Muscat et al., 1992) and are unaltered by disruptions to hippocampal neurogenesis (Bessa et al., 2009; David et al., 2009). Therefore the disruption of antidepressant efficacy by EndoN treatment is likely via the disruption of D_2 receptor expression resultant of attenuated synaptic plasticity. There is a known PSA-NCAM-D_2 receptor interaction, as D_2 receptor activation increases PSA-NCAM expression in the medial prefrontal cortex (mPFC) (Castillo-Gomez et al., 2008), while EndoN treatment attenuates D_2-mediated plasticity in the mPFC (Castillo-Gomez et al., 2011). Together these studies, along with the present findings, suggest an essential role for PSA-NCAM in the dopamine-mediated antidepressant effects of FLX which serve to reduce anhedonia.

In the forced swim test, infusion of EndoN prevented the FLX-mediated decrease in immobility, and increase in swimming, in CUS exposed rats. Serotonergic antidepressants increase swimming behaviour in the forced swim test which is dependent on activation of the 5-HT_{1A} receptor (Detke et al., 1995; Redrobe et al., 1996), and swimming behaviour is not affected by reductions in neurogenesis (Bessa et al., 2009; David et al., 2009). Thus, our findings suggest that the depletion of PSA-NCAM, via EndoN, interferes with appropriate serotonergic neurotransmission subsequently altering stress-coping behaviors in the forced swim test. Indeed, PSA-NCAM expression in the hippocampus is directly modulated by the 5-HT_{1A} receptor, and

conversely PSA-NCAM may play a role in regulating 5-HT receptor expression in the raphe nucleus (Brezun and Daszuta, 2000; Black et al., 2009; Grzegorzewska et al., 2010). The prevention of increased swimming by EndoN treatment is therefore likely indicative of altered serotonergic neurotransmission via the 5-HT_{1A} receptor. The glucocorticoid receptor is also important in the FLX-induced reduction in immobility (Lee et al., 2014) and glucocorticoid receptor activation is linked to hippocampal PSA-NCAM expression (Rodriguez et al., 1998). Our findings therefore demonstrate an essential role for PSA-NCAM in the antidepressant effects of FLX, potentially achieved through the modulation of serotonergic neurotransmission and modulation of glucocorticoid receptor activity, likely via downstream effects on neuroplasticity.

EndoN also prevented the FLX-mediated reduction in latency to feed in the novelty suppressed feeding test, representing a disruption of neurogenesis-dependent antidepressant effects; as attenuation of hippocampal neurogenesis also eliminates the ability of FLX to reduce the latency to feed (Santarelli et al., 2003; Bessa et al., 2009; David et al., 2009). It is possible that the effect of PSA-NCAM cleavage on novelty-induced hypophagia is due to a direct disruption of hippocampal neurogenesis, as found in the present study. Alternately, the depletion of PSA-NCAM may produce downstream effects via disrupted serotonergic neurotransmission which regulates adult hippocampal neurogenesis through the 5-HT_{1A} receptor (Radley and Jacobs, 2002; Banasr et al., 2004). Interestingly, EndoN treatment also disrupted the antidepressant effect of FLX in the noCUS treatment group, causing a significant increase in latency to feed. Acute treatment with FLX is anxiogenic (Silva et al., 1999), suggesting the ablation of PSA-NCAM and attenuation of the antidepressant effects of FLX may facilitate underlying anxiogenic properties. Taken together, our behavioural findings show that the ablation of PSA-NCAM, via EndoN treatment, impedes antidepressant efficacy in behavioural

measures dependent on neurogenesis (novelty suppressed feeding test) and behavioural measures independent of alterations in neurogenesis (sucrose preference and forced swim test), which instead, rely upon the modulation of monoaminergic neurotransmission.

4.4.3 EndoN reduced some, but not all, markers of neuroplasticity in the hippocampus

The infusion of EndoN functioned as intended, greatly reducing the expression of PSA-NCAM 7-weeks after administration. Our findings also show attenuated neuroplasticity, including the abatement of adult hippocampal neurogenesis by EndoN treatment, seen as a reduction in the number of BrdU-ir cells and BrdU/NeuN co-labelled cells 7-weeks after EndoN treatment, though cell proliferation (Ki67) was unaltered. This finding somewhat contrasts a previous report showing an increase in the proportion of BrdU/NeuN co-labelled cells, though cell proliferation and the number of 1-week old BrdU-ir cells were not significantly affected by EndoN treatment (Burgess et al., 2008). However, the length of the present study (53 days) greatly exceeds the previous report of 7-days, and suggests that EndoN reduces long-, but not short-term, survival of new neurons. EndoN reduced the expression of PSD-95 in the dentate gyrus and CA3 regions and to a lesser extent CA1, a finding indicative of reduced NMDA receptor expression and excitatory synaptic density (El-Husseini et al., 2000; Lin et al., 2006).

4.4.4 EndoN disrupted some, but not all, effects of FLX on neuroplasticity following CUS

EndoN infusion attenuated the effects of FLX on hippocampal neuroplasticity, including measures of synaptic density (PSD-95 expression), neural activation (cFos expression), and neurogenesis. FLX treatment increased PSD-95 expression in the stratum lucidum of CA3 and the dentate gyrus in a manner consistent with the literature which shows increased synaptic

density following antidepressant treatment in both regions (Reines et al., 2008). EndoN attenuated this FLX-induced enhancement of PSD-95 expression in CA3 and dentate gyrus. This is the first finding demonstrating the inhibition of the antidepressant-induced enhancement of synaptic density via the selective depletion of PSA-NCAM. Disruption of the behavioural antidepressant efficacy of FLX coinciding with the inhibition of the FLX-induced upregulation of structural remodelling observed in our study, is consistent with previous findings showing an essential role of such plasticity in antidepressant efficacy (Bessa et al., 2009).

Following acute stress, cFos expression in the dentate gyrus was increased in the noCUS groups compared to the CUS groups. Lower cFos expression in rats exposed to CUS implies a reduced level of neural activity likely due to habituation of the stress-response in the CUS rats (Willner, 1997). Interestingly, FLX treatment decreased cFos-expression regardless of stress, which may be representative of facilitated HPA negative feedback with FLX treatment (Lee et al., 2014). Importantly, EndoN inhibited the effects of FLX, but only in the CUS exposed group, serving to effectively increase neural activation in response to acute stress to the level of the noCUS groups.

4.4.5 EndoN treatment did not eliminate FLX effects on endocrine measures

Unlike both behavioural and neural responses to CUS, EndoN did not significantly influence basal CORT or testosterone levels in response to FLX or CUS. In the present study we found FLX treatment reduced serum testosterone levels but there was no significant effect of FLX to reduce basal CORT after CUS consistent with previous reports (Bataineh and Daradka, 2007). These findings suggest that while EndoN serves to mediate the behavioural efficacy of FLX, it failed to affect changes to the gonadal axis in response to FLX or CUS.

4.4.6 Conclusions

Our findings demonstrate that the depletion of PSA-NCAM inhibits the behavioural efficacy of FLX and some aspects of FLX-induced neuroplasticity, showing PSA is required for antidepressant action. Given the role of PSA-NCAM in modulating a broad range of plasticity, this glycoprotein functions at the crux of multiple antidepressant mechanisms, and subsequently antidepressant efficacy. Thus, PSA-NCAM represents an interesting target for future research into the development of novel pharmacotherapies.

5. GENERAL DISCUSSION

The research in this thesis aimed to investigate the role of androgens in the pathogenesis and treatment of depression using a CUS model of depression in male rats, with a specific eye toward the impact on hippocampal neurogenesis and neuroplasticity, and whether neuroplasticity mediated through PSA-NCAM is essential to antidepressant efficacy. The major findings of this thesis are that: (1) Gonadectomy of male rats potentiates the expression of depressive-like physiological, endocrine, behavioural, and neurological endophenotypes, including reduced hippocampal neurogenesis and expression of PSA-NCAM, in a 3-week CUS model of depression (Chapter 2: Wainwright et al., (2011)); (2) Testosterone treatment in gonadectomised male rats exposed to 3-weeks of CUS shows antidepressant-like action, and enhances the antidepressant effects of the TCA imipramine, in a state- and behaviour-dependent manner. Furthermore, treatment with the combination of testosterone and imipramine serves to increase the hippocampal expression of PSA-NCAM (Chapter 3: Wainwright et al., (resubmitted)); and (3) Depletion of PSA-NCAM via treatment with EndoN inhibits the efficacy of the SSRI fluoxetine in all behavioural measures and serves to reduce hippocampal neurogenesis and the expression of synaptic proteins (Chapter 4: Wainwright et al., (in press)). Taken together, the experiments in this thesis demonstrate that androgens modulate the susceptibility to the development of depressive-like endophenotypes in males, in a manner that coincides with alterations in hippocampal neuroplasticity, and demonstrates an essential role for neuroplasticity mediated through PSA-NCAM in antidepressant action.

5.1 Androgens Modulate Vulnerability to the Effects of Chronic Unpredictable Stress on Anxiety- and Depressive-like Endophenotypes

There is ample evidence in the clinical literature demonstrating the association of depression with hypogonadism in men. However, whether hypogonadism is a contributing factor in the pathogenesis of depression, or a symptom resultant of depression is less clear. The data presented in Chapter 2 demonstrates that the removal of testicular hormones potentiates the effects of CUS-induced depressive-like behaviours, reductions in hippocampal neurogenesis, and reductions in hippocampal PSA-NCAM expression. In short, I found that gonadectomy results in greater vulnerability to stress both in the development of depressive-like behaviours and effects on hippocampal plasticity. The findings presented in Chapter 2 therefore successfully model the comorbidity of hypogonadism and depression in men, and demonstrate concurrent declines in hippocampal plasticity.

It is important to note that the level of circulating testosterone is not the only factor in determining the influence of androgens in the vulnerability, or resistance, to depression. The CAG polymorphism dictates the sensitivity of the androgen receptor, and subsequently the feedback, and set point of circulating levels of testosterone (Crabbe et al., 2007). Individual differences in androgenic signalling and feedback may therefore influence the susceptibility to depression. In accordance with the stress diathesis model of depression, it is pertinent to suggest that some men may be more inclined to the development of depression due to alterations in androgens, or insensitivity of the androgen receptor. The efficacy of testosterone treatment in hypogonadal men may therefore be dependent upon the sensitivity of AR in relation to circulating testosterone levels, where men more vulnerable to depression are susceptible to alterations in testosterone levels (Seidman et al., 2001b).

Testosterone supplementation has shown antidepressant efficacy in hypogonadal depressed patients (Zarrouf et al., 2009). However, the means through which testosterone ameliorates depressive symptoms and facilitates antidepressant efficacy, is not well understood, nor had it been examined in a CUS model of depression. The data presented in Chapter 3 demonstrates that testosterone treatment in agonadal male rats exposed to CUS ameliorates HPA hyperactivity and the development of some depressive-like behavioural phenotypes, while also serving to enhance the behavioural efficacy of imipramine and increase the hippocampal expression of PSA-NCAM. The findings presented in Chapter 3 demonstrates that testosterone produces antidepressant-like effects alone, and aids antidepressant efficacy, via multiple mechanisms as elucidated through endocrine, behavioural, and neurological measures. I will next briefly discuss some of the possible downstream mechanisms of androgen action.

5.1.1 Androgen-mediated mechanism of action: Glucocorticoids

The data presented in Chapter 2 demonstrates that surgically gonadectomised male rats exposed to CUS show increased passive (immobility) behaviours compared to rats receiving either treatment alone. This finding is consistent with other studies, both prior and subsequent, showing that gonadectomy alone increases the time spent immobile in the FST and the latency to immobility, which is reversed by treatment with testosterone (Bernardi et al., 1989; Carrier and Kabbaj, 2012b). Exposure to CUS increases immobility behaviour in the FST in a manner that is directly associated with serum corticosterone levels (Baez and Volosin, 1994). Moreover, activity of hippocampal GRs directly influence the expression of immobility behaviour, as several studies using GR antagonists (Bachmann et al., 2005; Solomon et al., 2014), GR knockouts (Tronche et al., 1999), and antisense to hippocampal GR mRNA (Korte et al., 1996),

all produce reductions in the time spent immobile in the FST. These findings from Chapter 2 are consistent with studies examining the link between androgens and stress-reactivity in male rodents. Previous studies have shown that the removal of testicular hormones potentiates the stress response in males following acute stress (Viau et al., 1999; Viau et al., 2003), and testosterone replacement, or supplementation, is associated with a recovery of the stress response (Seale et al., 2004a). Indeed, in Chapter 2 I found that gonadectomised males that had undergone 3 weeks of CUS had slightly higher basal levels of corticosterone, indicating that testicular hormones exert a negative influence, not only on stress reactivity but also on basal corticosterone levels after chronic stress. The influence of testosterone in ameliorating the effects of chronic stress is less well delineated and thus the findings from Chapter 3, examining the influence of testosterone during CUS, are pertinent to the existing literature.

The findings presented in Chapter 3 demonstrate that in the FST the administration of testosterone alone increased the latency to the first bout of immobility and potentiated the antidepressant effects of imipramine when they were co-administered. Antidepressant drugs, including both SSRIs and TCAs, modulate GR activity in a manner that corresponds with decreased immobility behaviour in the FST (Anacker et al., 2011a), therefore the facilitation of antidepressant efficacy by androgen treatment may be a facilitation of this mechanism. Previous studies have shown that the administration of DHT decreases the expression of GR in the CA1 region of GDX males (Kerr et al., 1996). Androgens function to directly inhibit HPA output at the level of the hypothalamus (Viau and Meaney, 1996). However, depletion of GR in the PVN has no effect on FST behaviour in either male or female mice (Solomon et al., 2015). Though speculative, the mechanism through which androgens modulate behaviour in the FST may not be a direct effect in the hypothalamus, but mediated through androgenic modulation of hippocampal

GR expression. The data in Chapter 3 also show testosterone treatment significantly reduced basal corticosterone levels and adrenal weight, giving further support to a corticosterone or GR-mediated mechanism.

5.1.2 Androgen-mediated mechanism of action: Monoamines

The experiments conducted in Chapter 3 revealed no significant effect of testosterone treatment on the duration of swimming or struggling behaviour, and therefore no clear indication from the data presented in this thesis about the modulation of serotonergic or noradrenergic neurotransmission in the FST. However, this finding may be a consequence of the dose of testosterone used. Buddenberg et al. (2009) have shown a dose-dependent effect of exogenous testosterone in the FST in intact, non-stressed, male rats, where the 1mg/kg dose (as used in Chapter 3) failed to significantly decrease time immobile, while doses of 2mg/kg and 4mg/kg did. The influence of testosterone on active behaviours in the literature is also inconsistent, as increases in both swimming and struggling behaviour have been reported, although studies vary in the age of the subjects, the dose of testosterone, and the hormonal status of the subject prior to treatment (Buddenberg et al., 2009; Frye and Walf, 2009). Interestingly, one study observed that GDX blocked the antidepressant effects of the SSRI fluoxetine and the TCAs desipramine and clormiprimine, both derivatives of imipramine, in the FST. Testosterone supplementation only restored the antidepressant effect of desipramine, which produces potent inhibition of NE reuptake, therefore suggesting testosterone plays a key role in facilitating antidepressant effects mediated through noradrenergic neurotransmission (Martinez-Mota and Fernandez-Guasti, 2004).

Testosterone treatment facilitated the antidepressant efficacy of imipramine in the sucrose preference test, while neither treatment alone had a significant effect in Chapter 3 (Wainwright et al., resubmitted). Previous research has shown that chronic imipramine treatment increases sucrose preference following CUS exposure via a D_1/D_2-dependent dopaminergic mechanism (Muscat and Willner, 1989; Muscat et al., 1990; Papp et al., 1994). Testosterone can influence mesolimbic dopamine and response to reward (Hernandez et al., 1994), as male rats injected with testosterone, systemically or directly into the nucleus accumbens will develop a conditioned place preference (Packard et al., 1998). This testosterone-induced conditioned place preference is blocked by a dopamine antagonist (Packard et al., 1998). In fact, the injection of D_1 or D_2 antagonist inhibits the rewarding properties of testosterone, a mechanism that aligns with the antidepressant effects of imipramine (Schroeder and Packard, 2000). Therefore the results from Chapter 3 (Wainwright et al., resubmitted) suggest that testosterone facilitates the antidepressant action of imipramine on sucrose preference through the facilitation of D_1 and/or D_2 activation.

A study by Herrera-Perez et al., (2012) found that testosterone treatment alone failed to reverse the putatively anhedonic effects of CUS, but was only efficacious in the prevention of a CUS-induced decrease in sucrose preference. This finding may be indicative of long-term hypogonadism resulting in a down regulation of AR, and decreased sensitivity to androgen treatment (Kerr et al., 1995), as the study by Herrera-Perez et al., used 12-15 month-old male rats that had serum testosterone levels roughly a quarter of 3-5 month-old male rats. These studies suggest that androgens influence multiple aspects of monoaminergic neurotransmission, and likely exert, or facilitate, aspects of antidepressant action through this influence.

5.1.3 Androgen-mediated mechanism of action: Neuroplasticity

The data in Chapter 2 (Wainwright et al., 2011) show that gonadectomy served to potentiate CUS-induced reductions in hippocampal neurogenesis and PSA-NCAM expression. While testosterone treatment in Chapter 3 failed to enhance neurogenesis under CUS treatment conditions, it facilitated the enhancement of PSA-NCAM by imipramine treatment in CUS exposed rats. These findings demonstrate that androgens modulate hippocampal neuroplasticity, and facilitate antidepressant-induced neuroplasticity. This upregulation in hippocampal neuroplasticity may play a direct role in mediating antidepressant action.

The NSF test is unique, as antidepressant effects are only produced from chronic treatment and require hippocampal neurogenesis (David et al., 2009), therefore enhancements in hippocampal plasticity are directly related to latency to feed in this behavioural measure. The data in Chapter 3 demonstrate a state-dependent effect of testosterone serving to reduce the latency to feed in the NSF test following exposure to CUS (Wainwright et al., resubmitted). No mood improving effects of antidepressant treatment are typically observed in healthy individuals, suggesting that antidepressant action is dependent on the system in which it is acting (Harmer et al., 2003; Harmer et al., 2004). Indeed animal models of depression support this, as exposure to CUS has been shown to alter gene expression in corticolimbic structures including the dentate gyrus, while chronic antidepressant treatment reverses the CUS-induced alterations in gene expression in a manner that corresponds with the amelioration of depressive-like behavioural phenotypes (Surget et al., 2009). Importantly, chronic antidepressant treatment demonstrates state-dependent effects, as there is low similarity in the antidepressant-induced gene expression profiles of control versus CUS exposed mice (Surget et al., 2009).

Imipramine treatment reduced the latency to feed in both CUS and non-CUS treatment group, and there was no additive effect of testosterone and imipramine together. The role of hippocampal neurogenesis in the efficacy of imipramine in the NSF test has been established (Santarelli et al., 2003; Bessa et al., 2009), however the role of neurogenesis in the testosterone-induced reduction in latency to feed is less clear. We have previously observed increased cell survival in males receiving testosterone treatment (Spritzer and Galea, 2007; Hamson et al., 2013), however we did not see increased neurogenesis in the present study, consequently there is no evidence to suggest this process is required for the effects of testosterone within the NSF test. It is important to note that although we failed to see an enhancement of neurogenesis, neurogenesis still may play a essential role in the in the testosterone mediated antidepressant-like effects observed, as levels may still be sufficient to invoke antidepressant-like action. It is also likely that had testosterone treatment continued for another 9 days I would have observed an increase in neurogenesis as previously reported (Spritzer and Galea, 2007; Hamson et al., 2013). One way to examine whether immature neurons were increasing in number and may have contributed to the NSF findings in Chapter 3 would have been to examine a measure such as doublecortin. However, to truly elucidate the contribution of neurogenesis to the antidepressant-like effects of testosterone the NSF test would have to be conducted in rodents with ablated hippocampal neurogenesis using an irradiation, transgenic or pharmacological approach (Santarelli et al., 2003; Bessa et al., 2009; David et al., 2009; Snyder et al., 2011).

The NSF test is classically a test for anxiety-like behaviour and anxiolytic drugs, and treatment with exogenous testosterone produces anxiolytic effects in rodents (Aikey et al., 2002). Moreover, gonadectomy has anxiogenic effects in rodents, which are ameliorated by testosterone treatment (Carrier and Kabbaj, 2012a; Khakpai, 2014). The anxiolytic effects of testosterone are

blocked by the administration of the AR antagonist flutamide (Fernandez-Guasti and Martinez-Mota, 2005; Hodosy et al., 2012), suggesting the anxiolytic effects of testosterone are mediated by the AR. However, several metabolites of testosterone, including estradiol and 3α-diol, also produce anxiolytic effects independent of the AR (Frye and Edinger, 2004; Edinger and Frye, 2005; Carrier et al., 2015). Therefore, the reduction in latency to feed in the NSF test may not be due to a modulation of hippocampal neurogenesis but anxiolytic effect mediated in an AR-dependent or –independent manner.

5.2 Metabolites of Testosterone May Mediate the Antidepressant/Anxiolytic-like Effects of Testosterone Treatment

Testosterone is a precursor to several other bioactive steroid hormones; these include reduction to DHT, a potent androgen with high affinity for the AR, and aromatization to E_2, an estrogen that activates ER subtypes with high affinity. DHT can be further metabolized to 3α-diol, a metabolite with weak affinity for AR and ERβ, and 3β-diol a metabolite with affinity for ERβ (Goel et al., 2014). Therefore, the gonadectomy performed in Chapter 2 (Wainwright et al., 2011) did not simply disrupt AR-mediated mechanisms in response to stress, and thus the enhanced vulnerability to stress may be due to a paucity of estrogenic signalling. Similarly, the testosterone treatment in gonadectomised males utilized in Chapter 3 (Wainwright et al., resubmitted) may have conferred antidepressant-/anxiolytic-like effects and facilitated the antidepressant effects of imipramine via one of the bioactive metabolites. Treatment with testosterone was chosen for Chapter 3 (Wainwright et al., resubmitted), in order to align with the clinical literature, where the vast majority of studies use testosterone replacement therapies in hypogonadal men with comorbid depression (Zarrouf et al., 2009). However, it is important to

assess the potential contribution of steroids derived from testosterone in the potentiation of stress vulnerability in hypogonadal males, and in the antidepressant- and anxiolytic-like actions of exogenous testosterone treatment.

5.2.1 Estrogens produce antidepressant- and anxiolytic-like effects in males

The ER is expressed in the hippocampus of males rats at levels equal to that of female rats (Weiland et al., 1997). The activation of ER increases hippocampal spine density and rapidly modulates synaptic plasticity in male rats, demonstrating a functional importance for ER in hippocampal neuroplasticity (Gonzalez-Burgos et al., 2012; Hasegawa et al., 2015). Therefore it is reasonable to postulate the estrogens may exert, or facilitate androgenic, antidepressant- or anxiolytic-like effects in males.

There is evidence to suggest estrogens do in fact function to ameliorate depressive-like phenotypes in males. The duration of time immobile in the forced swim test in gonadectomised males is reduced by treatment with E_2, but not DHT (Carrier and Kabbaj, 2012b). This finding suggests that the antidepressant-like effects of testosterone treatment in gonadectomised males, as observed in Chapter 3 (Wainwright et al., resubmitted), occurs via an estrogenic mechanism via the aromatization of testosterone to E_2. Indeed, the aromatase inhibitor fadrozole, inhibits the antidepressant-like effect of testosterone to ameliorate a gonadectomy-induced reduction in sucrose preference, and prevents the anxiolytic effect of testosterone treatment in the OFT (Carrier et al., 2015). Similarly, the aromatase inhibitor formestane disrupts the antidepressant efficacy of both the SSRI fluoxetine and the TCA desipramine in the FST, in intact males. Conversely, E_2 treatment at physiological levels facilitated the efficacy of fluoxetine and desipramine in GDX males, and supra-physiological doses of E_2 alone produced antidepressant-

like effects in GDX males (Martinez-Mota et al., 2008). Both the Carrier et al., (2015) and the Martinez-Mota et al., (2008) studies demonstrate that the antidepressant like effects of testosterone treatment, alone and as an adjunct treatment facilitating antidepressant efficacy, as observed in Chapter 3 (Wainwright et al., resubmitted), are mediated through an estrogenic mechanism following the aromatization of testosterone to E_2. However, it is important to note that rats were not stressed in either the Carrier et al., (2015) or the Martinez-Mota et al., (2008) study, thus it is unclear if aromatase inhibitors would inhibit the antidepressant-like effects of testosterone alone or the enhancement of imipramine efficacy, if experiments were conducted in a CUS model of depression.

Importantly, no study has observed estrogenic modulation of antidepressant efficacy in the NSF test, nor independent effects of estrogen treatment in the neurogenesis-dependent NSF test, in male rats. Previous research from our lab has shown an AR-dependent mechanism for the enhancement of hippocampal neurogenesis (Spritzer and Galea, 2007; Hamson et al., 2013). However, as previously stated, the state-dependent effects of testosterone treatment in the NSF test may occur via an anxiolytic mechanism.

5.2.2 3α-diol has anxiolytic effects in male rodents

The metabolism of DHT by hydroxysteroid dehydrogenases (HSD) produces 3α-diol and 3β-diol. Both metabolites are biologically active steroids, however research has pointed to 3α-diol as a key factor in producing anxiolytic effects in male rodents. Treatment with DHT produces anxiolytic effect in both intact and gonadectomised male rats, an effect that is attenuated by the intrahippocampal infusion of indomethacin, a 3α-HSD inhibitor, suggesting DHT is metabolized in the hippocampus to 3α-diol which in turn exerts the anxiolytic effects. (Frye and Edinger,

2004). Furthermore, the systemic or intrahippocampal administration of testosterone, DHT, or 3α-diol all produce anxiolytic effects (Edinger and Frye, 2004, 2005). 3α-diol is a positive modulator of the $GABA_A$ receptor (Reddy and Jian, 2010), which represents a likely mechanism through whichv3α-diol produces anxiolytic effects. Interestingly, $GABA_A$ antagonists disrupt the anxiolytic effects of testosterone treatment, thus the anxiolytic effects of testosterone may be mediated by metabolization to 3α-diol, which modulates hippocampal GABAergic neurotransmission (Fernandez-Guasti and Martinez-Mota, 2005).

5.3 Antidepressant Efficacy on Relation to PSA-NCAM

The exact mechanism through which antidepressant drugs exert their effects has yet to be elucidated. Multiple lines of evidence demonstrate the contributions of various avenues of antidepressant action, including the modulation of monoaminergic neurotransmission, and the upregulation of neurogenesis (Hirschfeld, 2000; Sahay and Hen, 2007). Recently neuroplasticity theories of depression have emerged aiming to unify the antidepressant effects on neurotransmission, neurogenesis, neurotrophic factor expression, and alterations to the neural architecture throughout the brain (Pittenger and Duman, 2008; Wainwright and Galea, 2013). Indeed, studies have demonstrated the importance of neural remodelling and global plasticity, over single facets of antidepressant actions such as serotonergic neurotransmission or neurogenesis (Holick et al., 2008; Bessa et al., 2009). The experiments in Chapter 4 aimed to be the first to directly modulate a molecule required for multiple forms of neuroplasticity and assess the impact on antidepressant efficacy.

The findings in Chapter 4 demonstrate that the selective cleavage of PSA-NCAM inhibits the antidepressant efficacy of FLX in the FST, the sucrose preference test, and the NSF test. The

depletion of PSA-NCAM therefore disrupts multiple antidepressant mechanisms; both monoaminergic and neurogenic. Indeed, PSA-NCAM is expressed in the raphe and directly related to serotonergic neurotransmission (Brezun and Daszuta, 1999), associated with dopaminergic neurotransmission and the D_2 receptor (Castillo-Gomez et al., 2008), and functions in adult hippocampal neurogenesis (Burgess et al., 2008). Though speculative, it is possible that EndoN treatment inhibited antidepressant efficacy by inhibiting the enhancement of serotonergic and dopaminergic neurotransmission via the restriction of synaptic plasticity. The disruption of antidepressant efficacy in the NSF test may be tied to the PSA-NCAM-induced reduction in cell survival. However, the fluoxetine-induced enhancement of cell proliferation was unaffected by EndoN treatment, a finding consistent with a previous study (Burgess et al., 2008).

Interestingly, the depletion of PSA-NCAM alone did not produce a depressive-like phenotype in any of the behavioural measures, while a previous study shows that a $NCAM^{-/-}$ knockout produces a depressive-like phenotype (Aonurm-Helm et al., 2008), therefore demonstrating that depleting the polysialylation of NCAM does not alter basal behaviour like depleting the core protein. Similarly, baseline feeding behaviour and locomotor activity were unaffected by the depletion of PSA-NCAM. These findings of unaltered basal behaviour are important, as a non-specific ICV infusion of EndoN was used in Chapter 4 (Wainwright et al., in press), therefore PSA was degraded throughout the brain.

5.4 Caveats and Considerations

A CUS model of depression was used in each of the experiments presented in this thesis. A 3-week protocol was used in both Chapters 2 (Wainwright et al., 2011) and 3 (Wainwright et al., resubmitted), while a 6-week protocol was used in Chapter 4 (Wainwright et al., in press).

Though the length of CUS protocols is highly variable (Hill et al., 2012), a 3-week protocol was chosen for in Chapters 2 and 3 to ensure that the duration of CUS exposure was lengthy enough to alter hippocampal neurogenesis, and in order to assess the survival of mature neurons labelled with BrdU at the beginning of experimentation as some studies using less than 3-weeks do not produce reductions in hippocampal neurogenesis (Hill et al., 2012). Similarly, the 3-week protocol was used in Chapter 3, in addition to maintain consistency with Chapter 2. However, the experimental design in Chapter 3 served to assess whether treatment with testosterone could *prevent* the development of depressive-like endophenotypes, not reverse extant depressive-like endophenotypes. The findings of Chapter 3 are therefore in line with others showing testosterone treatment is able to prevent, but perhaps not reverse stress effects in gonadectomised males (Carrier and Kabbaj, 2012a; Herrera-Perez et al., 2012). Indeed, to truly assess the antidepressant-like effects of testosterone treatment, a protocol like that used in chapter 4 is needed. Rats would receive 3-weeks of CUS to induce a depressive-like state, then subsequently receive chronic testosterone treatment concurrent with a further 3-weeks of CUS prior to behavioural testing and tissue collection.

In Chapter 3, the 3-weeks of CUS treatment failed to induce the same level of depressive-like endophenotypes relative to the non-stressed control group as seen in Chapter 2. However, there are some important methodological differences between the non-stress groups in Chapters 2 and 3. Notably, the non-stress, non-hormone, non-imipramine treated rats in Chapter 3 still received daily subcutaneous injections of hormone vehicle (sesame oil) and intraperitoneal injections of imipramine vehicle (0.9% saline). A single daily injection of saline has been shown to induce depressive-like endophenotypes in some strains of rats, and represents a chronic stressor in and of itself (Izumi et al., 1997). Furthermore, all rats underwent gonadectomy which,

as demonstrated in Chapter 2, enhances the susceptibility to CUS and increased the expression of depressive-like endophenotypes. Therefore the non-stress control groups, against which the expression of depressive-like endophentypes in the stressed groups was assessed, where intrinsically different between Chapters 2 and 3, with controls in Chapter 3 likely demonstrating much greater endophenpypes of depression thereby muting the effects of CUS.

Testosterone treatment alone was expected to increase the survival of BrdU-labelled cells in Chapter 3, given previous findings (Spritzer and Galea, 2007; Hamson et al., 2013). However, we observed no independent effect of testosterone treatment on cell survival. The lack of a significant effect may be due to the length of testosterone treatment, as 21 days of treatment was used in Chapter 3, while previous studies that observed a significant increase in cell survival used 30 days of testosterone or DHT treatment (Spritzer and Galea, 2007; Hamson et al., 2013). The length of androgen treatment appear to play an important role in the enhancement of hippocampal neurogenesis.

The experimental design in Chapter 4 relied upon the effects of EndoN treatment, and therefore the depletion of PSA-NCAM expression, lasting for longer than 7 weeks. A previous study had shown that after the ICV infusion of EndoN PSA-NCAM levels were undetectable after 2 weeks, a small amount of PSA-NCAM was detectable after a month, and PSA-NCAM levels had almost returned to normal after 6 weeks (Seki and Rutishauser, 1998). However, the study by Seki and Rutishauser (1998) infused EndoN on postnatal day 1, a time of great neuroplasticity, and high levels of PSA-NCAM expression (Edelman, 1986). During the postnatal period PSA-NCAM is naturally down regulated, and ultimately restricted to areas of high plasticity in adulthood (Rutishauser, 2008). Furthermore, Seki and Rutishauser used a highly diluted form of EndoN, compared to the high-activity enzyme used in the experiments of

Chapter 4. As expected, PSA-NCAM levels were significantly depleted, although the expression of PSA-NCAM had clearly begun to return. Importantly, the antidepressant effects of fluoxetine were still inhibited. We did not observe any effect of EndoN treatment on cell proliferation, which is consistent with the literature (Burgess et al., 2008), however a contribution of newly re-expressed PSA-NCAM within the dentate gyrus cannot be ruled out.

The effects of the experimental manipulations used in this thesis, including the application of CUS, gonadectomy, testosterone treatment, ICV infusion of EndoN, and antidepressant treatment all extend far beyond the hippocampus. The alterations to hippocampal morphology, neurogenesis, and protein expression are surly just one facet of numerous effects taking place throughout the brain. In studies of the pathogenesis and treatment of depression attention also needs to be focused on brain regions such as the PFC and amygdala, which are both strongly implicated in depression. The assessment of brain regions outside of the hippocampus has been beyond the scope of this thesis, but that does not mean they have been discounted.

Lastly, all of these studies were conducted in male rats, and as outlined in Chapter 1 profound sex differences exist in the incidence and manifestation of depression, as well as antidepressant efficacy (Galea et al., 2013). Animal studies bear this out, as the vulnerability to, and the presentation of, depressive-like endophenoypes show definitive sex differences. For example, the presentation of depressive-like phenotypes following the depletion of TrkB (Monteggia et al., 2007) or forebrain GR (Solomon et al., 2012) is sex-dependent. Thus, the vulnerability and pathogenesis of depression in females is undoubtedly distinct from that of males. While the objective of the Chapters 2 and 3 of this thesis was to assess the influence of

androgens on the vulnerability of males to developing a depressive-like phenotype in a CUS model of depression, I recognize the male-centric nature of the research.

5.5 Future Directions

5.5.1 Beyond the hippocampus

The hippocampus was the region of focus for this thesis, as it demonstrates a great degree of plasticity in response to stress, primarily due to the high density of GR in the hippocampus (McEwen, 1973; Morimoto et al., 1996). However, the androgen-induced resilience to the development of depressive-like endophenotypes is likely mediated at multiple levels of interaction between the HPA and HPG axes. Therefore future experiments should assess the effects of androgenic signalling at the level of the hypothalamus in a CUS model of depression; in intact, gonadectomised, and testosterone treated males.

The use of ICV infusion of EndoN in Chapter 4 precludes the identification of any region-specific mechanism of antidepressant action being abated through the depletion of PSA. Future studies should investigate local infusions of EndoN into corticolimbic structures such as the PFC, amygdala, and the hippocampus, to determine the relative contributions of region-specific PSA-NCAM mediated neuroplasticity to antidepressant efficacy. Assessing the contribution of PSA-NCAM to the efficacy of other classes of antidepressant such as TCAs and serotonin-norepinephrine reuptake inhibitors (SNRIs) would also be prudent given the broader action of TCAs and the less well delineated role of PSA-NCAM in noradrenergic neurotransmission. Similarly, it would be interesting to examine the role of PSA-NCAM in the rapid antidepressant effects of ketamine (see section 5.5.2), given the associated rapid

enhancement of dendritic and synaptic plasticity in the hippocampus and PFC (Hayley and Litteljohn, 2013). The mechanisms underlying the influence of PSA-NCAM on antidepressant efficacy may be elucidated through the examination of receptor interactions and regulation, and ultimately lead to the development of novel pharmacotherapies.

5.5.2 Polysialic acid may have antidepressant efficacy via NMDA receptor antagonism

Ketamine is a non-selective NMDA receptor antagonist that serves to rapidly activate the mammalian target of rapamycin (mTOR) signaling pathway which is associated with the ketamine-induced antidepressant action (Maeng et al., 2008). The effects of ketamine are functionally replicated by a NMDA receptor NR2B subunit-specific antagonist, both in animal models and clinical studies, which represent a novel target for antidepressant intervention without the psychotomimetic effects of ketamine (Maeng et al., 2008; Preskorn et al., 2008; Li et al., 2010). Polysialic acid interacts with the NMDA receptor NR2B subunit in a highly-specific and antagonistic manner, which is mimicked by the NR2B-specific antagonist ifenprodil (Hammond et al., 2006). Therefore, the enhancement of the polysialylation of NCAM may result in antidepressant-like effects, and could represent a mechanism through which PSA-NCAM facilitates rapid antidepressant efficacy.

5.5.3 The modulation of BDNF signalling as a mechanism for the effect of PSA-NCAM depletion on antidepressant efficacy

Hindered BDNF signalling may be the downstream avenue through which the depletion of PSA-NCAM may disrupt antidepressant efficacy. Brain-derived neurotrophic factor (BDNF) has been implicated in the etiology and treatment of depression. Depressed patients show decreased levels

of serum BDNF (Karege et al., 2002; Shimizu et al., 2003), which is correlated with decreased hippocampal volume and increased ratings of depression (Shimizu et al., 2003). Conversely, increased BDNF levels are associated with antidepressant treatment and alleviation of depression (Aydemir et al., 2005). These findings are mirrored in rodent models of depression (Russo-Neustadt et al., 1999; Shirayama et al., 2002; Altar et al., 2003; Vinet et al., 2004; Gronli et al., 2006) as chronic antidepressant treatment increases BDNF levels, coinciding with alleviation of depressive like behaviours (Russo-Neustadt et al., 1999; Altar et al., 2003; Vinet et al., 2004) and intracranial infusion of BDNF produces antidepressant effects (Siuciak et al., 1997; Shirayama et al., 2002)

Activation of the TrkB neurotrophin receptor largely mediates the plasticity-enhancing actions of BDNF (Seil and Drake-Baumann, 2000; Danzer et al., 2002; Li et al., 2008), where inhibition of TrkB signalling attenuates the behavioural efficacy of antidepressants (Li et al., 2008). Conversely BDNF, or more specifically proBDNF, via activation of the p75 neurotrophin receptor mediates plasticity-reducing actions such as apoptosis, neural atrophy, and synaptic pruning (Castren and Rantamaki, 2010). It has been postulated that the balance of TrkB and p75 signalling may underlie antidepressant effects of BDNF, suggesting a mechanism through which stress/depression may modulate the effects of BDNF. To this point, knocking out TrkB receptors fails to produce a depressive-like phenotype in neurogenesis-dependent or –independent behavioural measures of antidepressant efficacy (Saarelainen et al., 2003; Zorner et al., 2003), however it is worth pointing out that sex differences exist, as female TrkB knockouts do appear to develop a depressive-like behavioural phenotype (Monteggia et al., 2007).

Interestingly, PSA-NCAM interacts with BDNF, as removal of PSA from NCAM inhibits the induction of LTP, the application of exogenous BDNF restores LTP at the affected

synapse (Muller et al., 2000). Further, disruption of the polysialylation of NCAM also disturbs the effects of BDNF on cortical neuron differentiation and survival, while the application of exogenous BDNF reverses these effects (Vutskits et al., 2001). These findings suggest that PSA-NCAM may mediate the responsiveness of neurons to BDNF. It is known that PSA-NCAM interacts with, and may regulate, p75 expression in septal neurons (Burgess and Aubert, 2006) and newly generated neurons of the SVZ (Gascon et al., 2007b). Indeed knockout of p75 significantly reduces the expression of PSA-NCAM in SVZ neuroblasts (Young et al., 2007). As previously mentioned the p75 receptor is involved in the regulation of adult hippocampal neurogenesis (Catts et al., 2008) and regulates neurogenesis stimulated by chronic antidepressant treatment (Colditz et al., 2010). Given the complex and varied role of BDNF in the etiology of depression and antidepressant treatment more research is needed to further elucidate a mechanism through which the modulation of BDNF exerts its influence via PSA-NCAM.

5.5.4 The PSA mimetic compound 5-Nonyloxytryptamine as a potential antidepressant

The 5-HT_{1B} receptor varies in function from inhibitory, to excitatory, or as an autoreceptor, based on the region of the brain it is expressed in (Zhuang et al., 1999). Research has indeed shown that 5-HT_{1B} receptor knockout mice show increased anxiety (Zhuang et al., 1999), while some 5-HT_{1B} receptor agonists show antidepressant efficacy, including the recently approved antidepressant drug vortioxetine, which acts as a partial agonist (Pearce and Murphy, 2014). 5-Nonyloxytryptamine oxalate (NNT) is a selective 5-HT_{1B} receptor agonist that, importantly, is inactive against the 5-HT_{1A} receptor (a primary target of most antidepressant drugs). Interestingly, NNT also functions to mimic polysialic acid, in both structure and function, via the interaction with NCAM. Though a much smaller molecule than PSA, NNT is able to adopt a

shape compatible with the van der Waals volume of the PSA-binding conformation, as confirmed through the interaction with a PSA-specific monoclonal antibody. Moreover, NNT is able to functionally mimic PSA through the interaction with NCAM. For example, NNT induces neurite outgrowth, decreases cytotoxicity and apoptosis, and stimulates cell migration in cultured primary neurons (Aonurm-Helm et al., 2008). The application of NNT also increases the expression of NCAM and PSA-NCAM, thereby facilitating endogenous neuroplasticity. The importance of such a mimetic compound, and the potential production of others, lies in their metabolic stability and ability to cross the blood-brain barrier to reach the desired targets compared to generating and administrating purified PSA. Compounds that can directly stimulate and induce neuroplasticity, such as NNT, represent what may be an exciting new class of drugs that provide rapid and long-lasting antidepressant effects.

5.6 Conclusions

The experiments presented in this thesis demonstrate that androgens confer resiliency to the development of depressive-like endophenotypes (Chapter 2, 3; Wainwright et al., 2011; Wainwright et al., resubmitted) and facilitate the antidepressant efficacy in a manner that coincides with increased PSA-NCAM expression, which is required for antidepressant efficacy (Chapter 4; Wainwright et al., in press). However, the means through which androgens reduce stress reactivity and ameliorate the effect of chronic stress on hippocampal morphology are unclear, as numerous active metabolites of testosterone function to reduce depressive-like endophenotypes through multiple mechanisms, including via the AR. Though the contribution of testosterone-mediated enhancements in hippocampal neuroplasticity to a reduced vulnerability to developing depressive-like endophenotypes is unknown, it is clear that antidepressant treatment

requires PSA-NCAM mediated neuroplasticity. Future studies should build off of these findings and investigate the means though which PSA-NCAM enables antidepressant efficacy, and assess the potential for PSA-NCAM as a novel drug target.

In conclusion, it is imperative that future studies account for the multiple factors of influence on the resilience, or vulnerability to, stress and depression. These factors, as determined by the experiments in this dissertation are gonadal status, testosterone levels, and the capacity for neuroplasticity. It is recommended that studies examining the underlying mechanisms of depression utilize chronic, rather than acute conditions, such as the one employed in this dissertation (CUS), particularly in consideration for potential state-dependent treatment effects. These types of studies are vital to the improvement of reproducibility in animal research and to the validity of animal models of human disease such as depression.

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