SEX, STRESS AND HORMONES: INVESTIGATING THE ROLE OF THE EARLY ENVIRONMENT IN SHAPING BRAIN AND BEHAVIOUR CHANGES LINKED TO NEUROPSYCHIATRIC DISORDERS

by

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ABSTRACT

Vulnerability to anxiety and depression are linked with both sex differences and early life stress. Though human studies suggest that childhood symptoms and neurophysiological markers may predict some adult neuropsychiatric disorders, few animal models examine early developmental time-points when studying effects of stress and/or sex differences, especially as forecasters of later disease. In this thesis, effects of an ethologically relevant model of prenatal predator stress (PPS), with/without postnatal enhanced housing (EHC) was explored in a rat model of anxiety/depression. First, behavioural changes following PPS/EHC were quantified in both juvenile and adult rats on tasks modeling anxiety and depression. Adult males exposed to PPS demonstrated increased anxiety and some disrupted social behaviours, which were partially rescued by EHC. Following PPS, adult females exhibited anhedonia that was prevented by EHC. Effects of EHC were more prominent in juveniles, while many effects of PPS emerged in adulthood. Juvenile behaviours did not correlate directly with equivalent adult behaviours, but some juvenile behaviours predicted other adult anxious/depressive behaviours. In subsequent studies, changes in an epigenetic marker (DNMT3a) or a GABA marker (GAD67) were quantified in the brains of juveniles exposed to either the PPS/EHC paradigm, to examine its role in disrupting early development, or to postnatal androgens, to examine the role of organizational hormones in establishing sex differences at this time. In both males and females, PPS acted primarily to increase DNMT3a-ir, though there were sex differences in which regions were affected, and whether EHC could 'rescue' these changes. Sex differences in DNMT3a-ir were found in some brain regions, and androgens partially mediated these differences. Effects of PPS on GAD67-ir were dependent on sex, decreasing GAD67-ir in males and increasing it in females, in different brain regions. Though EHC prevented/rescued many of these changes, it also independently altered GAD67-ir in several regions in females. Sex differences in GAD67-ir were primarily found in the amygdala, with a complex role for organizational androgens in establishing these differences. Together, these studies demonstrate sex-specific changes in both brain and behaviour at an early developmental time point, which may predict some adult vulnerabilities to anxiety and depressive behaviours.

LIST OF ABBREVIATIONS USED

3βADiol – 5α- androstane 3β, 17β Diol
5-HT – 5-hydroxytryptamine, serotonin
ANOVA—analysis of variance
AR – androgen receptor
ATD – 1,4,6-androstatriene-3,17-dione
AVP – arginine vasopressin
BDNF – brain derived neurotrophic factor
BLA – basolateral amygdala
BNST – bed nucleus of the stria terminalis
BPA – bisphenol A
BSA – bovine serum albumin
CeA – central amygdala
CORT – corticosterone
CpG – cytosine-guanine dinucleotide
CRF—corticotrophin-releasing factor
DES – diethylstilbestrol
DEX – dexamethasone
DHT – dihydrotestosterone
DMH – dorsomedial hypothalamic nucleus
DNA – deoxyribonucleic acid
DNMT – deoxyribonucleic acid methyltransferases
ED – embryonic day
EE – enriched environment
EHC – enhanced housing condition
ER – estrogen receptor
FLU - flutamide
fMRI – functional magnetic resonance imaging
FSL – Flinders Sensitive Line
FST – forced swim test
GABA—gamma-aminobutyric acid
GAD – glutamic acid decarboxylase
GC – glucocorticoid
GD – gestational day
GFP – green fluorescent protein
GNX – gonadectomy
GR – glucocorticoid receptor
HDAC – histone deacetylase
HIER – heat-induced epitope retrieval
hCG – human chorionic gonadotropin
HPA—hypothalamic-pituitary-adrenal
HPG – hypothalamic-pituitary-gonadal
IHC – immunohistochemistry
IR – immunoreactive
KCC2 – potassium-chloride co-transporter 2

LG - licking and grooming
LPS – lipopolysaccharide
MDD – major depressive disorder
MR – mineralocorticoid receptor
MRI – magnetic resonance imaging
NAc-C – nucleus accumbens core
NAc-Sh – nucleus accumbens shell
NC – naïve control
NGS – normal goat serum
NKCC1 – sodium-potassium-chloride-co-transporter 1
OF—open field test
PBS – phosphate-buffered saline
PFA – paraformaldahyde
PFC – prefrontal cortex
PLP – pyridoxal 5'phophate
PND – postnatal day
PPS – prenatal predator stress
PTSD – post-traumatic stress disorder
PVC - polyvinyl-carbonate
PVN—paraventricular nucleus of the hypothalamus
SC – standard cage
SHRP – stress hypo-responsive period
SPSS—Statistical Package for Social Sciences
SRY – sex-determining region of the Y chromosome
TfM - testicular feminization mutation
TMS – transcranial magnetic stimulation
USV - ultrasonic vocalizations
WKY – Wistar Kyoto

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CHAPTER 1 - INTRODUCTION

Neuropsychiatric disorders, including anxiety and depressive disorders, are increasingly recognized as a leading cause of disability worldwide and a major contributor to global disease burden (Ferrari et al., 2013). According to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric Association, 2013), to be diagnosed with a major depressive disorder patients must display at least five of the symptoms described below on most days (for a minimum of two weeks), including at least one of the two hallmark symptoms of the disorder - depressed mood or anhedonia (a loss of pleasure or interest in daily activities), along with: decreased energy and activity levels, disturbed sleep patterns, changes in appetite, inability to concentrate, feelings of worthlessness or guilt, pessimistic thoughts, and thoughts of suicide or self-harm. Anxiety symptoms that may co-occur with depression include irrational worry, preoccupation with unpleasant worries, trouble relaxing, or a fear that something awful might happen. Increasingly, research has also focused on changes in social behaviour, including lower levels of extraversion, reduced affiliative behaviour, social withdrawal, and a lower motivation to seek social support, as both a risk factor for depression and a common marker of depressed individuals (Hames et al., 2013; Cruwys et al., 2014; Girard et al., 2014).

Despite the high incidence of these disorders, a coherent model of the underlying disease processes causing anxiety and depression has yet to be developed, and available drug treatments are not effective in a number of patients (Nestler et al., 2002). Depression is a complex, multifaceted illness, and different clusters of symptoms are present within

individual patients, such that there may be very little clinical overlap between two individuals with the same diagnosis of 'depression' (Andrews et al., 2007). Depressive disorders are often categorized into two distinct subtypes, with sets of symptoms of opposing valence. In melancholic depression, patients show a loss of appetite, insomnia, anxiety, and increasing activation of the stress response system, while with atypical depression, patients present with fatigue, excessive daytime sleepiness, increased appetite, and (according to some studies), a down-regulated stress response (reviewed in Gold and Chrousos, 2002; Antonijevic, 2006; 2008). To better target interventions, a more complete grasp of the disease processes, the underlying neural mechanisms, and possible sources of vulnerability that result in different symptoms are required. Improved models of anxiety/depressive symptoms using non-human animals, especially those relating to key markers of depressive behaviour including despair, anhedonia, and social withdrawal, will play an important role in this process (Anisman and Matheson, 2005; Matthews et al., 2005). The complex etiology and symptom presentation of major depressive disorders lends support to the idea that their development is based on a combination of factors, including genetic predisposition, gonadal hormones, significant early life events, and exposure to triggering events in adulthood (reviewed in Farabaugh et al., 2004; Hammen, 2005; Marcus et al., 2008). In this work, I have chosen to focus on behavioural and neural changes in early life that may contribute to the later development of anxiety or depressive disorders.

The possibility of different underlying pathophysiology being associated with different disorder subtypes highlights the important question of what is being modeled in rodent tests of anxiety or depression. As described above, depression is a complex,

multifactorial illness, and it is unlikely that any one model will reproduce all the behavioural deficits of the disorder; particularly as different clusters of symptoms are present within individual patients. Further, there are several symptoms (feelings of guilt, thoughts of self-harm, etc.) that cannot be measured, or perhaps even exhibited, in rodents for obvious reasons. There are, however, certain agreed-upon criteria for an animal model to be considered a valid representation of an aspect of depression. These have been discussed in several recent reviews (Krishnan and Nestler, 2011; Kumar et al., 2013; Andrews et al., 2014; Molendijk and de Kloet, 2015; Czéh et al., 2016), and limitations of some tests will be considered again in Chapter 7, thus I will only mention briefly here that good models require: 1) face validity – apparent subjective similarities in the symptoms or behaviours being modeled, 2) predictive validity – recovery or attenuation of the aforementioned symptoms with treatments that are effective in humans, 3) etiological validity – symptoms can be induced by the same types of events thought to be important in the human condition 4) construct validity – behavioural changes are induced by similar changes to the brain as those seen in the disorder, and 5) pathophysiological validity – similar underlying changes to physiology are found in the model and the disease. Notably, both 4 and 5 are somewhat difficult to corroborate in animal models, as a full understanding of the underlying neurobiological changes in depression are not yet known. When developing rodent models of depression, the particular tests used to assess the symptoms and behaviours being characterized are also an important consideration, as a variety of tests exist to model each of the different clinical symptoms (reviewed in Czéh et al., 2016). I will briefly touch on the behavioural

tasks chosen for use in this thesis, and discuss the rationale for their selection, though limitations will be discussed in more detail in Chapter 7.

The forced swim test (FST) is one of the most commonly used tests in the rodent depression literature, and was first developed by Porsolt and colleagues in the 1970s as a test to screen antidepressant effectiveness (Porsolt et al., 1977). In this task, rats or mice are placed in a container filled with enough water that they cannot touch the bottom, and must stay afloat. Initially they try to escape by swimming, diving, or climbing the walls of the container, but over time the animal becomes increasingly immobile, floating rather than continuing to attempt escape. The amount of time spent immobile and/or the latency before this immobility develops, has often been considered a marker of behavioural 'despair', or a decrease in active coping, and analogous to depressed mood. A number of commonly used antidepressants can prevent or decrease this immobility, giving the model good predictive validity, though the interpretation of the behaviour as 'despair' rather than an adaptive learned response is often debated in the literature (Molendijk and de Kloet, 2015). Many studies have demonstrated that severe acute stress or chronic mild stress in adulthood, or stress in early life, can significantly decrease the latency to first immobility or increase the duration of immobility, though this result is not found consistently across both sexes or all stressor paradigms (reviewed in Donner and Lowry, 2013; McHenry et al., 2014; Santarelli et al., 2014; Bock et al., 2015; Boersma and Tamashiro, 2015; Cartier et al., 2015).

Although the FST was developed for adult animals, in recent years researchers have demonstrated that rat pups as young as postnatal day (PND) 21 show significant dose-dependent effects of antidepressants on immobility behaviour. Notably, the

particular antidepressants that were effective or ineffective in this model were similar to those that were effective in childhood depression (Reed et al., 2008; 2009), suggesting predictive validity of the FST in juvenile animals as well as adults. Following acute restraint stress, PND21 rats, like adults, show increased FST immobility (Bernal-Morales et al., 2009) further supporting the use of this test in pre-pubertal animals.

Anhedonia is considered one of the most important endophenotypes of depression as it is highly heritable and may be a stable 'trait' marker for vulnerability to depression, even outside of a current depressive episode (Pizzagalli, 2014). In rodents, this is most often modeled using a sucrose preference task (SPT), where the preference for a sucrose solution (usually 1-2%) is calculated compared to a neutral solution (water) in a two bottle choice paradigm. Rats typically show a strong preference for the sucrose solution when it is provided alongside water. Sucrose preference, rather than sucrose intake, is thought to be a more sensitive measure of anhedonia as it controls for changes in overall fluid intake, and the decreased preference for sucrose over water is interpreted as a decreased hedonic value, or less 'pleasure' being derived from consuming the sweet solution (reviewed in Rygula et al., 2005; Heshmati and Russo, 2015). Sucrose preference often decreases following chronic stress, corticosterone injections, or early life stress (Jayatissa et al., 2006; Sáenz et al., 2006; Strekalova et al., 2006; Mueller and Bale, 2007; Rygula et al., 2008; Bitanihirwe et al., 2010) though this effect is not consistent across all 'depressive' paradigms or between the sexes (Baker et al., 2006; Green et al., 2009; Behan et al., 2011; Carrier and Kabbaj, 2012a; Van den Hove et al., 2014). As intake of sucrose is a caloric activity, it is impossible to completely separate the 'pleasure' component of the action from the consumptive act (Anisman and Matheson,

2005; Heshmati and Russo, 2015), though some studies with saccharin (a non-caloric sweetener) have shown similar patterns of response to stress and antidepressants (Pijlman et al., 2003; Grønli et al., 2006; Malkesman et al., 2006; Czéh et al., 2016). As chronic stress can also result in sustained weight loss and diminished food intake, there is a concern that the reduced preference for sucrose following stress might reflect this decreased consumptive behaviour or lower caloric needs. This concern is partially addressed both by calculating preference for sucrose as a percentage of total fluid intake (rather than simply using total volume of sucrose solution consumed), as well as calculating sucrose preference relative to the body weight of the animal being tested (Hill et al., 2012). One major caveat of the sucrose preference test, however, is that, unlike models of intracranial self-stimulation, some researchers have suggested that sucrose preference may not accurately model the motivation and reward seeking aspects of anhedonia, but merely the hedonic value of the stimulus (Anisman and Matheson, 2005; Heshmati and Russo, 2015; Czéh et al., 2016). However, as discussed in detail in recent reviews, the relative roles of motivation and pleasure in clinical anhedonia is itself not well understood, and thus the importance of this distinction to models of anhedonia are not known and perhaps not essential to our understanding (Heshmati and Russo, 2015; Czéh et al., 2016).

As described previously, dysfunctional social behaviour is another important component of major depressive and anxiety disorders, and some researchers have focused on models of social interaction or avoidance as a measure of rodent 'depression' as well (Heshmati and Russo, 2015; Czéh et al., 2016). Research has focused primarily on the late juvenile and adolescent periods, as active social play behaviours are most abundant

during this time, and they exhibit a characteristic developmental pattern that can be examined in a laboratory setting. In rats, social play develops throughout the juvenile period, peaking in early adolescence (Panksepp and Lahvis, 2007). Juvenile rats will learn to traverse a T-maze to obtain access to a playmate at the other end (Humphreys and Einon, 1981), demonstrating that social play is itself a rewarding experience for juvenile rodents. Depression-related anhedonia can therefore potentially be assessed in juvenile rats by studying the hedonic effects of social play, with the added face validity that children with depression tend to exhibit social withdrawal, social impairment and social skills deficits including reduced eye contact, failure to initiate conversation, and rejection by peers (Karevold et al., 2012; Silk et al., 2012; Marwick et al., 2013). Studies of rodent juvenile play behaviour involve observing a defined period of social interactions, with either a familiar or unfamiliar conspecific, and observing the duration and/or latency of established 'normal' play behaviours. Malkesmen and colleagues have examined the behavioural and neural profiles of pre-pubertal rats using genetic models of adult depression (Flinders Sensitive Line – FSL, and Wistar Kyoto – WKY). These strains demonstrate many of the symptoms associated with childhood and adult depression, including altered sucrose preference anhedonia and forced swim immobility, as well as disruptions to social play behaviour and adult social interactions. These behavioural alterations are accompanied by brain changes (including disruptions to monoamine levels) and changes to the hypothalamic-pituitary- adrenal (HPA) axis, a major pathway involved in the physiological response to stress, which are often altered in depression and anxiety (discussed in more detail in Section 1.1.2; reviewed in

Malkesman and Weller, 2009), suggesting these tasks may be useful for studying juvenile depression.

Anxiety and depressive disorders show a high rate of co-morbidity in both children and adults, and the co-occurrence of these disorders often results in a worse long-term prognosis than if anxiety or depression are diagnosed alone. There is also considerable overlap between diagnostic criteria for generalized anxiety disorder and major depression in children, and some researchers therefore suggest the two should not be studied in isolation (Silk et al., 2012). Models of anxiety are typically based on either a conditioned response to a painful, stressful or fear-inducing situation, or on a spontaneous natural response to stressful stimuli that are not painful (Kumar et al., 2013). One commonly used measure of anxiety using exploration and locomotor behaviour is the Open Field test (OFT; Choleris, 2001). The OFT takes advantage of the natural neophobia of rodents, and evaluates anxiety-like and defensive behaviours in a novel open environment, creating a conflict between motivation to explore and the instinctive fear of open, well lit areas where they are more vulnerable to predation. This paradigm has face and construct validity as a test for anxiety-related behaviours in adult rats, and has been pharmacologically validated by studies of putative anxiolytics (for review, see Andrews et al., 2014). The Open Field has also been successfully used to assess effects of prenatal stress at PND10-15 (Mychasiuk et al., 2012b), amphetamines at PND15 (Laviola et al., 2004b), anxiety behaviors in response to acute stress in PND22 rats (Bernal-Morales et al., 2009) and postnatal flutamide at PND27 (Zhang et al., 2010a). Interestingly, one study demonstrated that following a prenatal developmental insult (exposure to lead) increased ambulation was found in the OFT, but not another

commonly used anxiety test, the Elevated Plus Maze, in PND23 rat pups (Moreira et al., 2001), suggesting that the OFT may be a more sensitive test of early life developmental changes in these younger animals.

Although there are a number of other behavioural tests that model these same, and other, symptoms of anxiety and depressive disorders (reviewed in Krishnan and Nestler, 2011; Czéh et al., 2016), I chose to focus on these particular tests (FST, OFT, Social Interaction, Sucrose Preference) as they model some of the core symptoms of depression (anhedonia, 'despair', disrupted social behaviours) and co-morbid generalized anxiety. Further, as discussed in detail through the rest of this Chapter, there is a growing need for better developmental and childhood models of anxiety/depressive disorders, and these tests are some of the most well used at early developmental time points. Certainly, none of these behavioural tasks are perfect models of human clinical disease, and limitations of these tasks will be discussed in Chapter 7, but they are a promising place to start. In particular, I chose to focus on behaviour in juvenile rats beginning with the OFT at PND15, as (in addition to our own pilot studies) this was one of the youngest ages with a behavioural response to enrichment or amphetamine treatments in the literature (Laviola et al., 2004b; Mychasiuk et al., 2012b). As will be discussed throughout this Chapter, there is also a need for a better understanding of the role of both sex differences and the process of sexual differentiation in the early life 'programming' of developmental vulnerability to anxiety and depressive disorders. PND15 is also thought to be a time point that falls at the end of the critical period for sexual differentiation (reviewed in Mccarthy and Nugent, 2015; Tsai et al., 2015), making it a particularly interesting initial

age to study the intersection of juvenile anxiety/depressive behaviours and sexual differentiation (discussed in Section 1.4).

1.1. Developmental Origins of Health and Disease

Clinically, neuropsychiatric disorders can manifest at many different time points across the lifespan. However, substantial evidence exists demonstrating that the early environment can serve to permanently shape the brain, contributing to the development of a range of later adult diseases, including anxiety and depressive disorders (Heindel et al., 2015). The idea that interactions between the fetal genome and the early environment can cause long-term changes in the brain and behaviour that manifest as adult disorders is known as the Developmental Origins of Health and Disease (DoHaD) theory. This idea first came to prominence in the late 1980s in studies of British cohorts, where regional variations in heart disease mortality (and later other metabolic diseases) were found to correlate with infant mortality rates, which in turn, appeared to be driven by regional differences in low birth weight (Barker, 2007). Since then, the field has expanded rapidly, presenting a number of associations between early life events such as prenatal malnutrition, maternal illness, and early life stressors, on diseases that emerge later in life, from obesity and heart disease to neuropsychiatric disorders including schizophrenia, anxiety and depression.

Although the DoHaD theory links fetal and early life developmental events to adult outcomes, it has become increasingly clear that there are often observable markers of disorders that are present prior to adulthood that also have their origins in early life (Gaffrey et al., 2013). A growing body of evidence suggests that adult anxiety/depressive disorders can be predicted by the presence of even subclinical childhood symptoms

(Moffitt et al., 2007; Shankman et al., 2009; Wesselhoeft et al., 2013), along with markers such as increased sensitivity to interpersonal stress or emotions (Rudolph and Flynn, 2007; Kovacs and Lopez-Duran, 2010), difficult infant tempermanent (Côté et al., 2009), or physiological changes such as altered EEG and MRI responses to emotional stimuli (Feng et al., 2011; Gaffrey et al., 2011; Pagliaccio et al., 2012; Das et al., 2013). Children diagnosed with emotional and behavioural problems also share many (age-adjusted) clinical symptoms with adolescent and adult anxiety and depressive disorders, as well as significant continuity with later childhood and adolescent episodes, suggesting these may be early-onset forms of disease, rather than a separate clinical entity (Mason et al., 2004; Pagliaccio et al., 2012; Leyfer et al., 2013). Indeed, clinical evidence suggests that sub-threshold pediatric anxiety or depressive symptoms and low positive affect in childhood could predict subsequent Major Depressive Disorders (MDD; reviewed in Garber, 2006; Shankman et al., 2009; Kovacs and Lopez-Duran, 2010). Infant temperament at 5 months has also been demonstrated to be a significant predictor of preschool anxiety and depressive symptoms (Côté et al., 2009) though much research remains to be done to ascertain whether these very early behavioural markers are true prodromal precursors of disease, or sources of increased vulnerability. Beyond behavior, there are a number of physiological changes in children with anxiety and depressive symptoms that reflect some of the changes seen in later adult depressive episodes. Compared to controls, children with severe clinical depression are more likely than controls to show increased activation to sad faces in prefrontal and limbic regions of the brain (Barch et al., 2012), along with altered stress responses (Suzuki et al., 2013), and changes in patterns of neural activity while in negative moods (Pagliaccio et al., 2012).

Together, these changes may reflect an altered developmental trajectory, and an increased vulnerability to later anxiety or depressive disorders (Lewis et al., 2014). Some longitudinal birth cohort studies have suggested that the majority of adult disorders are preceeded by their juvenile counterparts, either in the same diagnostic category or from another disorder (Hofstra et al., 2002; Reef et al., 2010). As anxiety/depressive disorders are being increasingly viewed as neurodevelopmental in origin, there is emergent interest in the identification of early risk factors and patterns of onset. This could help improve age-specific early diagnostic thresholds, as well as enhance existing models of disease development, and provide new targets for early treatments or even preventative interventions (Beesdo-Baum et al., 2015; Stirling and Toumbourou, 2015). In addition to a rising incidence of childhood anxiety and depressive disorders (Belfer, 2008; Rapee et al., 2009; Leyfer et al., 2013), children with anxiety/depressive symptoms also tend to have worse long-term outcomes than those whose symptoms only manifest in adulthood, including higher rates of other co-morbid neuropsychatric disorders, obesity, and substance abuse problems (Mason et al., 2004; Rapee et al., 2009; Reef et al., 2010; Batterham et al., 2013; Korczak et al., 2013; Queen and Ehrenreich-May, 2013), providing further incentives for early intervention. Together, this has led to a recent surge in research focusing on the expression of anxiety/depressive disorders across early development.

The importance of early patterns of brain and behaviour development in predicting and treating neuropsychiatric disorders that manifest in adolescence and adulthood highlights the need for more research directed at these early time points in both clinical and animal models. A better understanding of the developmental trajectory of

anxiety/depressive disorders, including identifying unique childhood symptoms and neural pathologies, will prove invaluable to our understanding of the etiology of these disorders, and help to create a better framework for treatments and interventions - perhaps even providing opportunities to begin intervention before serious clinical disorders can develop. In recent years, work has focused on adolescence as a sensitive period for environmental insults, as well as a critical period for shaping the 'typical' development of the brain (Romeo, 2010; Wright and Perrot, 2012; Bayless et al., 2013; Mccormick and Green, 2013; Morrison et al., 2014), but there is still a lack of animal models of pre-pubertal behavioural and brain changes related to mood and anxiety, particularly following early life stressors, as will be discussed in detail below.

1.1.2. Prenatal Stress and Anxiety/Depressive Disorders

Exposure to stress has been established as a key risk factor in the development of adult anxiety or depressive disorders, with an abundance of studies showing that stress during adulthood can trigger a depressive episode, while stress early in life can significantly increase vulnerability to developing these disorders in adolescence and adulthood (reviewed in Duman, 2010; Schmidt, 2011; Glover and Hill, 2012; Morley-Fletcher et al., 2012; Pizzagalli, 2014). Briefly, the stress response can be defined as the set of behavioural, physiological, endocrine and neural changes that occur in response to any stimulus that is perceived to be threatening or challenging to an organism's homeostasis (Selye, 1950). Appropriately responding to these stressors is a necessary part of survival for all organisms. When a stressor is perceived, a number of physiological systems are activated, but it is not the intent of this chapter to discuss them in detail (for a review, see Charmandari et al., 2005). The HPA axis is the 'final common pathway' of

this stress response system, and is a key component of regulating homeostasis in the face of a stressor. There are a number of limbic and prefrontal regions, including the amygdala, hippocampus, and prefrontal cortex, which act in concert with hypothalamic nuclei and subcortical sites, including the bed nucleus of the stria terminalis (BNST) and dorsomedial hypothalamic nucleus (DMH), to assess and process stressors, resulting in the potential activation of an HPA response. The HPA response begins with release of corticotrophin releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus and results in release of glucocorticoids (GC; ex. corticosterone, cortisol) from the adrenal gland. GCs act on almost every tissue and organ system, having the overall effect of releasing energy sources from storage (ex. enhancing gluconeogenesis and lipolysis) and inhibiting energy storage and physiological functions like feeding and reproduction (Charmandari et al., 2005). In addition, glucocorticoids pass through the blood-brain barrier to activate receptors involved in their own negative feedback response, including the type I mineralocorticoid receptors (MR), involved in maintaining basal HPA activity, and the Type II glucocorticoid receptors (GR), which are involved in negative feedback when GC levels are higher, such as in response to a stressor (reviewed in Herman and Cullinan, 1997; Herman et al., 2003; de Kloet, 2004). Dysfunctional CRF release, as a result of dysfunctional activation of the HPA axis, has been implicated in the pathophysiology of both anxiety and depressive disorders (reviewed in Bale, 2005; Binder and Nemeroff, 2010; Xiong and Zhang, 2013). While some aspects of the stress response system are conserved across species (Yao and Denver, 2007; Yao et al., 2008), others may vary, even within primates, and certainly across different mammalian groups (Sapolsky, 2003). Thus, it is important that any model of the human stress response is

fully characterized with respect to various outcome measures, including behavioural, hormonal, and neural changes. Many factors contribute to the high degree of individual variability in stress responding, as well as to the long-term effects of repeated exposure, including life history, and biological factors such as sex and age. Sex and sex steroids also modulate responses to stressors, as well as outcomes of stressor exposure and these findings have implications for reported sex differences in the incidence of stress-related diseases such as depression (reviewed in Altemus et al., 2014; Young and Pfaff, 2014).

Stressors during early life exert more potent and long-lasting effects on both brain and behaviour than equivalent stressors during adulthood. Moreover, stress during particular sensitive periods of development, when the brain is susceptible to 're-wiring' by the environment, can result in long-term programming of the brain. Depending on which brain structures are undergoing maturational changes, and therefore may be particularly vulnerable to environmental influences, and at which stage the developmental trajectory is disrupted, different physiological or behavioural changes can result (reviewed in Kofink et al., 2013; Bock et al., 2014; Lewis et al., 2014). Prenatal stress in particular, exerts long term effects on both physiology and behavior - disrupting adult HPA function, and contributing to vulnerability to many neuropsychiatric disorders, including depressive and anxiety disorders, in both humans (Hines et al., 2002; Van den Bergh et al., 2005; 2008; Davis and Granger, 2009; Kleinhaus et al., 2013) and non-human animal models (reviewed in Glover and O'Connor, 2002; Goel and Bale, 2009; Charil et al., 2010). Interestingly, one longitudinal study in humans shows a direct correlation among maternal prenatal anxiety, altered cortisol profile in adolescent offspring, and greater depressive symptoms in female adolescents (Van den Bergh et al.,

2008). In humans, prenatal stress can alter aspects of infant HPA response and temperament (reviewed in Austin et al., 2005), and maternal co-morbid anxiety and depressive disorders appear to significantly increase circulating cortisol levels and decrease cognitive abilities in infants (Azak, 2012; Azak et al., 2013), children (O'Connor et al., 2005), and in young adolescents (MacMillan et al., 2009). Anxiety in the third trimester is strongly linked to behavioural/emotional problems in 4-year-old boys and girls, even after controlling for postnatal anxiety and depression in the mother (O'Connor et al., 2002). Similarly, children whose mothers have anxiety/depression also show prepubertal brain changes, including a decreased nucleus accumbens activation in response to reward (Gotlib et al., 2010), increased amygdala activation to threat (Monk et al., 2008), as well as decreased hippocampal volume (Campbell et al., 2004; Pagliaccio et al., 2014), all of which could increase vulnerability to later neuropsychiatric conditions (discussed further in Section 1.2; (De Bellis and Keshavan, 2003; Brunton and Russell, 2011).

In addition to the timing of the stress exposure, the intensity, type, and duration of the stressor (reviewed in Lupien et al., 2009) interacts with underlying individual differences to determine the ultimate neural and behavioural changes that result from stress. Sex differences during development are one example of an underlying individual difference that contributes to the diverse effects of stress on the development of neuropsychiatric disorders. There are significant sex differences in the incidence of anxiety/depressive disorders in adulthood, with females having a higher prevalence than males. Sex differences are also found in symptom profiles in clinical studies (reviewed in Silverstein, 2002; Altemus et al., 2014), as well as in some of the effects of early life

stress and maternal depression on later vulnerability to depressive behaviours (reviewed in Weinstock, 2007; Glover, 2014; Graignic-Philippe et al., 2014). These differences appear to depend in part on the timing of prenatal stress exposure: clinically, some studies have reported that male children are more sensitive to effects of early maternal stress or anxiety, while females are more sensitive to later stress (Rice et al., 2007; Van den Bergh et al., 2008; de Bruijn et al., 2009; Pagliaccio et al., 2015). In a mouse model, Mueller and Bale (2008) also found that stress exposure in early development preferentially alters stress reactivity and neurotransmitter systems in males, but not females, while a number of other studies have found that stress during the last week of gestation in rat models can have a range of sex-specific effects on adult behaviours (reviewed in Zuena et al., 2008; García-Cáceres et al., 2010; Van den Hove et al., 2014). Together, these results suggest that the developmental trajectories involved in shaping sex differences in the brain, both in 'typical' development and following prenatal stress, may contribute to vulnerability to later anxiety or depression. A more detailed review of sex differences in early development will be discussed in **Section 1.4.**

In addition to sex differences and timing of exposure, the intensity of stress exposure, its duration, predictability, and underlying 'type' are all believed to play a role in the long term changes associated with prenatal stress. Many of the stressors used to induce stress in rodent models (foot shock, repeated restraint, swim stress) have a strong physical component, while many of the stressors experienced in humans are primarily psychological (Abe et al., 2007). Further, the ethological validity of a stressor (illness, predator, etc.) could differentially alter a number of aspects of the maternal stress experience, as well as the behavioural responses to these threatening stimuli (Heiming et

al., 2009). One recent study demonstrated that physical and psychological prenatal stressors have unique, and sex-specific, effects on adult cognitive and motor behaviours (Nazeri et al., 2015). Given this potential variability, a variety of psychological stress paradigms are needed in order to more accurately model the different aspects of the human prenatal stress experience, and as discussed in Chapter 2, 3, and 5, we believe that using an ethologically relevant, and unconditioned, exposure to predators could provide important new information about the diverse ways in which prenatal stress can impact the developing brain and behaviour.

1.1.3. Factors mediating the response to Early Life Stress

Beyond the timing, intensity, and type of stress exposure, early life stress is likely further mediated by a number of other factors – as where some individuals demonstrate vulnerability, others show resilience, even to the same stressful experiences (Parker and Maestripieri, 2011; Wright and Perrot, 2012; Boersma and Tamashiro, 2015). The recently introduced "integrated hypothesis of programming effects", states that some subjects may be at greater risk for psychopathology when life stress accumulates, while others are at greater risk if a 'mismatch' occurs between their early and later environments, suggesting that an individual's genetic makeup, their sensitivity to early programming, and their later environment, will all contribute to their individual vulnerability to anxiety/depression (Nederhof and Schmidt, 2012).

One factor that may be contributing to differences in the effects of prenatal stress are differences in the stress response of individual mothers. For example, recent studies using rat lines bred for either high or low anxiety, high or low novelty responsivity, or specific depressive phenotypes, suggest that exposure to early life stress can have quite

different, and sometimes opposite, effects on anxiety or depressive behaviours in offspring of these different dams (Frye and Wawrzycki, 2003; Malkesman et al., 2008; Neeley et al., 2011; Richetto and Riva, 2014; Río-Ålamos et al., 2015). Some of these differences may be due in part to different levels of maternal glucocorticoids in response to stressors, or differences in activation of enzymes at the placenta in response to circulating glucocorticoids (Cartier et al., 2015; Hinde et al., 2015). Typically, levels of glucocorticoid exposure in the fetus are lower than in the mother. However, chronic exposure to prenatal stress has been shown to disrupt an enzyme known as 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) which normally acts at the feto-placental barrier to metabolize active GCs and minimize fetal exposure to circulating maternal GCs. Disruption of this barrier by stress alters fetal GC exposure (reviewed in Gheorghe et al., 2010; St-Pierre et al., 2015). The response to stress is known to vary between individual dams, even within strains, and these maternal differences in levels of circulating GC, either initially or in response to changes in 11βHSD2 function, could be contributing to differences in the long-term effects of stressors.

Prenatal stress is also likely interacting with cues in the early postnatal environment to help program long-term vulnerability to anxiety or depression. Maternal care, for example, is known to alter aspects of stress responding in rodent models (Meaney, 2001). Higher levels of maternal care can act to prevent some of the effects of prenatal stress or exogenous glucocorticoids on offspring development (reviewed in de Kloet et al., 2014), and cross-fostering of offspring to control dams, or additional postnatal handling, can prevent some, but not all, of the effects of prenatal stress (Ábrahám and Kovács, 2000; Barros et al., 2004; Rice et al., 2009; Cirulli et al., 2010;

Pérez-Laso et al., 2013; Wattez et al., 2014). Further, prenatal stress may itself alter aspects of maternal behaviour (Smith et al., 2004; Champagne and Meaney, 2006), as prenatal exposure to high, but not low, doses of corticosterone (CORT) injections decrease maternal care (less nursing, less time spent on the nest; Brummelte and Galea, 2010). In addition to direct maternal care, the mother may also be altering offspring development via changes in lactation behaviour or hormonal signals (Koldovský et al., 1995; Walker, 2005). A number of important compounds and hormones are present in maternal milk, including cytokines, enzymes, prolactin (PRL), leptin, corticosterone, etc., and these compounds may be transmitting information about stressors in the maternal environment to pups during early lactation (Melo, 2015). Exposure to chronic stress may alter nursing patterns as well as directly increasing CORT exposure in pups via compounds in the milk (Zagron and Weinstock, 2006; Pereira et al., 2015). Interestingly, injecting mother rats with postnatal CORT results in elevated levels of CORT in the stomach milk and brain, but not plasma, of pups on PND7, and in plasma, but not the brain, on PND18, suggesting age- and tissue-specific effects of maternal milk hormones on development (Brummelte et al., 2010).

In addition to the direct effects of maternal care and milk, other cues in the early environment, such as environmental enrichment or isolation from the dam, can significantly impact developmental processes in the same systems that are targeted by prenatal stress. For example, brief, daily periods of maternal separation (usually 15 minutes or less), often called early handling, can result in changes to the HPA axis, such as lower CRF mRNA in the PVN, increased GR in the hippocampus, and a blunted GC response to stress, as well as altering monoamine systems and anxiety behaviours,

relative to non-handled animals or animals separated from their mother for longer periods of time (Levine, 1994; Meaney et al., 2000; Macrí et al., 2004; Pondiki et al., 2006; Stamatakis et al., 2006; Río-Ålamos et al., 2015). Importantly, though these brief separations do alter some aspects of maternal care, they also appear to act through independent mechanisms to direct HPA axis development (Macrí et al., 2004; Macrě et al., 2008). Similar to the effects of early handling, changes to the early environment through juvenile or adolescent environmental enrichment may prevent some of the long-term effects of prenatal insults (Morley-Fletcher et al., 2003; Welberg et al., 2006; Qian et al., 2008; Horvath et al., 2013; Peña et al., 2013) and accelerate aspects of neural development (He et al., 2010; Lonetti et al., 2010). However, these effects seem to depend on the timing of enrichment (Cutuli et al., 2011; Takuma et al., 2011), and may be sensitive to sex differences, as both early life enrichment and brief maternal separation may themselves be mild stressors, and are often administered during the period of sexual differentiation (discussed in Section 1.4). Recent research suggests that mild 'inoculating' stressors such as early environmental enrichment can act to prevent some of the effects of prenatal stress, or promote resilience in the face of later adult stressful experiences (Bock et al., 2015; Connors et al., 2015; Crofton et al., 2015), though there is a relative paucity of research including female subjects, and existing studies suggest sex is an important factor in the effectiveness of enrichment (Girbovan and Plamondon, 2013; Connors et al., 2014). One study found that low, but not high, dose injections of CORT in the postpartum period resulted in lower fear response to novelty and a stronger resistance to bacterial infection, in adult male mice compared to both control and high CORT groups, supporting the idea that low levels of corticosterone stimulation (and therefore

'inoculation' levels of stressful environmental enrichment in the early postnatal period) can be beneficial (Macrì et al., 2007). A better understanding of how postnatal environmental cues may interact with prenatal stressors to prevent or counteract the effects of early life stress, or independently promote resilience, across both sexes could provide important information about the ongoing early developmental processes involved in creating a vulnerability to anxiety/depression, as well as suggest potential targets for treatment.

Surprisingly, these postnatal environmental influences on the stress response and behaviour occur despite this period often being referred to as the stress hyporesponsive period (SHRP). Decreased levels of circulating GCs, as well as an increased resistance to HPA axis activation of ACTH or CORT by environmental cues characterize this period, usually defined in rats as PND3-PND14. The SHRP is believed to be critical to the development of the HPA axis, and while there appear to be mechanisms in place to minimize sensitivity to some disruptions (reviewed in Wiedenmayer et al., 2003; Faturi et al., 2010; Iwasa et al., 2012), certain types of stressors, such as predator exposure, extended maternal separations, or maternal abuse, have been shown to 'override' the SHRP blunted HPA response to cause a number of long-term changes in the brain and behaviour (Wiedenmayer et al., 2005; Lajud et al., 2012; Takase et al., 2012). Thus, it appears that the HPA response to stress may be functional at birth, but that the SHRP is maintained by 'normal' maternal cues (warmth, milk, maternal care, maternal environment), and disruptions to these cues are important regulators of the SHRP to help program later stress sensitivity and behavioural changes.

1.2. Brain Regions Involved in the Effects of Early Life Stress on Depression

Beyond the HPA axis and behavioural changes, it is important to understand the impact of early life stress on some of the brain regions that may be involved in the development of anxiety/depressive disorders. This may help us to understand both the developmental time course of observed behavioural and HPA-axis changes, and the neural pathologies underlying the later vulnerability to these disorders. Prenatal stress has been linked to changes in brain regions including the prefrontal cortex (PFC) (Muhammad et al., 2012; Dong et al., 2015; Soztutar et al., 2015), amygdala (Zhou et al., 2013; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015), nucleus accumbens (NAc; (Muhammad et al., 2012; Said et al., 2015), and hypothalamus (Zuloaga et al., 2012b), in both animal and human studies (reviewed in Charil et al., 2010). It should be noted here that this is by no means a complete list of all the brain regions that may be involved in the functional circuitry of depression (for a recent review of candidate brain region, see Pandya et al., 2012; Russo et al., 2012, Figure 1.1). Indeed, the complexity of emotion regulation and cognitive processes involved in generating even the individual symptoms of depression makes tracing all components of these pathways and their reciprocal connections difficult. I have chosen to focus on the regions discussed below (prefrontal cortex, nucleus accumbens, paraventricular nucleus of the hypothalamus, central amygdala and basolateral amygdala) as they have all been implicated in both human and animal studies of depression (See Figure 1.1), and, as discussed in subsequent sections of this Chapter, also show promise as targets where sex differences, early life stress, and epigenetic or GABAergic changes may intersect. With the understanding that there are

likely other brain regions, and other neurotransmitter systems, involved, we can begin to examine the role of these regions in anxiety/depressive behaviours.

The PFC is believed to be an important coordinator of the anticipatory response to stress, as well as goal-directed behaviour (McKlveen et al., 2015). The medial PFC has important connections with a number of limbic regions, including the ventral hippocampus, BNST and amygdala, as well as inputs to the serotoninergic and dopaminergic systems, making it a likely player in both stress regulation, and in vulnerability to neuropsychiatric disorders (reviewed in McLaughlin et al., 2014). Clinically, changes in both structure and function in the mPFC have been linked to depression in a number of large studies and meta-analyses (Videbech, 2000; Lemogne et al., 2012; Sacher et al., 2012; Lener et al., 2016), and sex differences in PFC volume have been related to differences in emotional processing, anxiety, and affect (Welborn et al., 2009). Indeed, even in non-clinical samples decreased grey matter volume of the dorsal mPFC is correlated with increased depressive traits in men, but not women, (Carlson et al., 2015). Similarly, postmortem studies have found a number of abnormalities in the PFC in patients with depressive disorders and in cases of suicide, including reduced cortical volume, changes to genes involved in glutamate and GABA receptors, as well as altered levels of neurotrophic factors, and decreased glucocorticoid receptors (Zhao et al., 2012; Goswami et al., 2013; Hayley et al., 2015; Zhao et al., 2015; Lener et al., 2016) reviewed in (Mcewen, 2015). Some of these changes appear to be sex-specific, with depressed females showing a greater upregulation of glutamate receptor genes in the PFC (Gray et al., 2015) and reduced levels of the brain-derived neurotrophic factor (BDNF;

(Hayley et al., 2015), suggesting that sex is an important factor in depression-related brain changes in this region.

The PFC is one of the later developing structures in the brain, and its structure and connectivity appear to be sensitive to the impacts of sex and the early environment from prenatal time points through the adolescent period (reviewed in Wright and Perrot, 2012; Hammerslag and Gulley, 2015), making the region particularly vulnerable to developmental disruptions and early stressors. In animal models, early life stress has been found to exert numerous effects on the structure and function of the adolescent and adult mPFC (Darbra and Pallarès, 2011; Muhammad and Kolb, 2011; Muhammad et al., 2012), particularly in response to acute stressors encountered as adults (Soztutar et al., 2015). Though the majority of studies have focused on the long-term effects of early life stress in the adult mPFC, some research has begun to investigate the role of earlier developmental changes that might contribute to later behavioural dysfunction (Murmu et al., 2006; Mychasiuk et al., 2011a; 2011c; 2012a). Indeed, recent research has demonstrated significant sex- and region-specific effects of prenatal restraint stress in the PFC, including increased spine density and increased number of excitatory synapses in the male mPFC (but not orbitofrontal cortex) where females showed a corresponding decrease in these parameters (Mychasiuk et al., 2011a), and both sexes showed increased synaptophysin density (Gemmel et al., 2015) by PND21, pointing to an important role of early development of the PFC in the response to prenatal stressors, and potential later vulnerability to neuropsychiatric disorders.

The NAc is an important component of the brain's reward processing system, with important connections to the mesolimbic dopamine pathway and motivational

processing (Cartier et al., 2015; Loke et al., 2015). The role of the NAc in the corticostriatal dopamine pathway is particularly interesting, as dysfunction in dopamine in this pathway is thought to play a key role in both motivation and reward processing, as well as in major depressive disorders (Ikemoto and Panksepp, 1999; Dunlop and Nemeroff, 2007; Der-Avakian and Markou, 2012). Patients with major depressive disorders demonstrate reduced activation in the NAc during a rewarding task, as well as altered connectivity with the ventral tegmental area, compared to healthy controls (Redlich et al., 2015). Similarly, in non-clinical populations, increased levels of anhedonia have been associated with reduced NAc volume and lower fMRI response to rewarding tasks (Wacker et al., 2009), suggesting an important role for the NAc in this key symptom of depression.

The NAc, like the PFC, continues to develop across childhood and adolescence, making it an important target both for early life stressors and potentially for early life interventions (Galvan et al., 2006). One clinical study has found that children with a history of severe childhood stress present with abnormalities in the development of the NAc during adolescence, and that these abnormalities correspond to higher rates of anhedonia (Goff et al., 2013), suggesting an important role for early life stress in developing adolescent depression via disruptions to this system. Another recent study has shown that, even after accounting for other depressive symptoms and recent stressful life events, an interaction between early-life stress and anhedonia appears to be moderated by blunted reactivity in the ventral striatum to reward (Corral-Frías et al., 2015). In rodent models, prenatal stress disrupts adult dopamine receptor expression in the NAc (Said et al., 2015), while prenatal and early postnatal stress alter aspects of the endocannabinoid

and dopaminergic systems, as well as modify dendritic morphology (Romano-López et al., 2015), spine density and dendritic branching (Muhammad et al., 2012), in the NAc, all of which could point to a link between changes in the NAc following prenatal stress and vulnerability to depression. Little research to date has focused on earlier (pre-adolescent) changes in the development of the NAc, but given the impact of both prenatal and postnatal stress, there may be important changes to this structure already present in the postnatal period.

The PVN, as described briefly in **Section 1.1.2**, is the 'final common pathway' for the brain's response to stress, triggering the HPA axis response via the release of CRF (Charmandari et al., 2005). One postmortem study has found that the number of CRF expressing neurons in the PVN was significantly higher in depressed subjects compared to healthy controls (Raadsheer et al., 1994). In a small study of seven depressed men and seven controls, postmortem analysis of the PVN revealed significantly increased CRF mRNA, as well as increased expression of CRF receptor 1, ERα, AVP and MRs, while ARs were significantly decreased in CRF neurons (Wang et al., 2008). In rodent studies, prenatal stress alters the GC response of the HPA axis to stress in adulthood, and these changes correlated with increased expression of CRF mRNA in the PVN in females, and decreased CRF binding protein (CRF-BP), which may limit CRF action, in males (Zohar and Weinstock, 2011). Studies in animal models have also linked changes in output from the PVN with anxiety behaviours (Norrholm et al., 2005; Blume et al., 2008). Interestingly, disrupting the embryonic development of the GABA system from gestational day (GD) 11-17 (a time frame which overlaps with stress exposure in many prenatal stress models), alters 'typical' sex differences in PVN GABA receptors,

and disrupts adult anxiety, hyperactivity, and depressive behaviours in a sex-specific manner (Stratton et al., 2014), suggesting a potential link between the sex-specific effects of prenatal stress on behaviour, and disruptions of the developmental trajectory of the PVN.

Clinically, the amygdala has been repeatedly associated with major depressive disorders in fMRI and volumetric studies. Differences in amygdala volume in human MRI studies have been linked to polymorphisms in genes related to serotonin transmission (Li et al., 2015a), suggesting a role for this neurotransmitter system, often linked to depressive disorders, in the development of the structure of the amygdala. A recent study has found links between traumatic or stressful life events, gene profiles associated with stress reactivity, and altered hippocampus and amygdala volumes in children, demonstrating an interesting connection between early life stress, genetic vulnerability, and early brain development (Pagliaccio et al., 2014). Another study by the same group has linked these polymorphisms in stress-associated genes with activation in response to emotional faces. Notably, the predictive effects of these gene polymorphisms on negative emotional stimuli were sex specific and found after, but not before, puberty, highlighting an important role of sex-specific pubertal development in the amygdala and the development of emotion-processing pathways (Pagliaccio et al., 2015). Adolescents diagnosed with depression have heightened activity in the amygdala in response to emotional stimuli, with less functional connectivity between the amygdala and mPFC when attempting to modulate and reduce these emotional reactions (Perlman et al., 2012). Remarkably, one recent study has found that preschool children with depression also show increased amygdala activation when viewing negative faces, while another

demonstrated altered connectivity between the amygdala and prefrontal regions in childhood onset MDD, suggesting that developmental changes in the amygdala related to depression may already be pathological at a very young age (Gaffrey et al., 2011; Luking et al., 2011; Gaffrey et al., 2012). Further, high maternal cortisol levels in early, but not later, pregnancy have been linked to smaller amygdala volumes in female, but not male, children, suggesting sex specific and stress-related contributions to these developmental changes (Buss et al., 2012).

Importantly, the more detailed spatial resolution available in postmortem studies, as well as in animal models, suggests that different nuclei of the amygdala may be uniquely contributing to anxiety and depressive disorders. In a recent postmortem study, markers of neuroplasticity were altered in the central nucleus of the amygdala (CeA) and basolateral amygdala (BLA) of depressed patients compared to healthy controls; notably, however, the effects in these two nuclei were in contrasting direction (Maheu et al., 2013). Postmortem research has also found increased levels of the D4 subtype of dopamine receptors (Xiang et al., 2008), a G-protein coupled dopamine receptor subtype associated with novelty seeking, (Lusher et al., 2001), as well as increased numbers of neurovascular cells, in the BLA, but not other subnuclei, in subjects with MDD compared to controls (Rubinow et al., 2014).

The CeA, along with the BNST, is a major source of CRF (outside the PVN), and is important in regulating the HPA axis via connections to the PVN, though there may be important species differences in CRF expression in this region (Asan et al., 2005). The CeA is also important in the expression of anxious behaviours, as well as fear learning (Keifer et al., 2015). Overexpression of CRF in the CeA increases anxiety behaviours in

rodent models (Flandreau et al., 2013), alters negative feedback of the HPA axis, and increases depressive behaviours on the forced swim test (Keen-Rhinehart et al., 2009). Interestingly, prenatal stress in particular alters CRF parameters in the CeA in a sex-specific manner, and these changes are associated with increased anxiety behaviour (Brunton et al., 2011; Zohar and Weinstock, 2011).

The BLA is important for emotional responses, and emotional memories, and well connects to the CeA and BNST to help mediate anxiety or fearful behaviours (Rodrigues et al., 2009; Walker et al., 2009), and to the NAc to modulate reward responses (Britt et al., 2012). The BLA is an important structure linking chronic stress with the development of anxiety disorders (Boyle, 2013), and both overexpression of CRF in the BLA, and alterations to GABAergic signaling in the BLA, have been linked to anxiety behaviours (Truitt et al., 2009; Hale and Lowry, 2010). In adolescent females, prenatal stress increased OFT anxiety behaviours and altered chloride co-transporter levels in the BLA – suggesting a change in GABAergic function in this region (Ehrlich et al., 2015), discussed further in Section 1.5). Prenatal stress also decreased socioemotional behaviours in male pups (reducing separation-induced ultrasonic vocalizations) and decreased adult male social interactions, which also correlated with alterations to adult BLA electrophysiology (Ehrlich and Rainnie, 2015). The impacts of early life stress on the BLA are likely connected to disruptions of developmental processes in these regions. Indeed, amygdala lesions on PND7 have severe effects on social behaviour, open field anxiety, and stress responding in adult animals, while later lesions (on PND21) have milder impacts on social behaviour alone (Wolterink et al., 2001; Daenen et al., 2002). Interestingly, connections between the BLA and PFC continue to mature through

adolescence, and disruptions to these connections have been hypothesized to relate to vulnerability to neuropsychiatric disorders (Cunningham et al., 2008; Casey et al., 2010; Somerville and Casey, 2010; Somerville et al., 2010), further highlighting the importance of the developmental trajectory of this brain region in social and anxiety behaviours.

In addition to development within these regions, connections between these brain regions may also play an important role in depressive symptoms. One study has found that a subset of patients with high levels of anhedonia, and correspondingly high levels of inflammatory markers in the blood also suffered from lower levels of connectivity and communication between the ventral striatum and ventromedial PFC (Felger et al., 2015). Similarly, regulation of the HPA axis (via the PVN) through negative feedback is also provided by the hippocampus and medial PFC, while the amygdala provides positive regulation of PVN (reviewed in Charmandari et al., 2005), and disruptions to these connections could contribute to disruptions in HPA axis responses following prenatal stress. Research has also linked a reduction in depressive symptoms following both placebo and antidepressant treatment in patients with major depression to opioid activity in the NAc, PFC, and amygdala, further pointing to the role of these regions in both symptoms of major depressive disorders, and targets for treatment (Peciña et al., 2015).

Taken together, these studies suggest that early life stress impacts a number of brain regions important for emotion, stress, and social regulation, which may be linked to development of neuropsychiatric disorders. Given the timing of these early life insults, and the diverse effects of disruptions across different developmental time points, it is clear that more research into the mechanisms underlying developmental changes to the structure and function of the immature brain, both in 'normal' conditions and in response

to stress, are necessary to understand the etiology of developing neuropsychiatric disorders.

1.3. The Role of Epigenetic Changes in the Effects of Early Life Stress

Early life stress may impact later offspring health by directly modifying the expression patterns of a gene, or its products. Epigenetic changes cause stable alterations to gene expression or function without altering the structure of the DNA itself, through mechanisms such as DNA methylation and histone modification. These changes are a crucial part of development, and are involved in differentiation of cell types, genomic imprinting, and growth and aging. Epigenetic changes are also sensitive to environmental influences, particularly in early life, and may be carried forward through development, and often passed on to subsequent generations. Thus, they are a likely candidate mechanism for some of the long-term 'programming' changes of gene expression observed in prenatal stress (reviewed in Essex et al., 2013; Kofink et al., 2013; Lester et al., 2013; Heindel et al., 2015; Jawahar et al., 2015). Moreover, epigenetic changes have recently been linked to anxiety and depression, among other neuropsychiatric disorders (reviewed in Jessen and Auger, 2011; Murgatroyd and Spengler, 2012; Kigar and Auger, 2013; Vialou et al., 2013; Menke and Binder, 2014).

1.3.1. Overview of Epigenetic Mechanisms

Although there are a variety of possible epigenetic modifications to the genome, including modification by noncoding RNAs such as microRNAs (miRNAs), the most well studied changes are in DNA methylation and histone modification, which are described briefly below. DNA methylation regulates chromatin structure, chromosome inactivation, genomic imprinting, and gene transcription. DNA methylation involves the

binding of methyl groups to cytosine, creating 5-methyl-cytosine (5MeC), primarily at CpG dinucleotides, though some recent research has demonstrated methylation of cytosines at non-CpG dinucleotide sites (Ito et al., 2010; 2011; Lister et al., 2013). Primarily, methylation appears to be an effective means for "silencing" of the area, by disrupting transcription factor binding or by altering the chromatin formation via recruitment of other proteins associated with repression, such as methyl-binding domain protein (MeCP2) or modification of histones, such as histone deacetylases (HDACs; (reviewed in Bird and Wolffe, 1999; Wolffe and Matzke, 1999; Moore et al., 2013).

The enzymes involved in establishing and/or maintaining these altered methylation patterns are known as DNA methyltransferases (DNMTs). DNMT1 is considered particularly important in maintaining preexisting patterns of methylation as it shows a preference for hemi-methylated sites, and is important for the inheritance of methylation patterns through cell division, though it may also contribute to some de novo methylation (Cheng and Blumenthal, 2008). DNMT3a and 3b are considered important for de novo methylation, laying down initial methylation marks. DNMT3b is thought to be expressed early on in neuroprogenitor cells, while DNMT3a is expressed in post mitotic, newly differentiated cells, and may play a larger role in cellular maturation and differentiation (Feng et al., 2005). Interestingly, though both enzymes function throughout the lifespan, DNMT1 and DNMT3a are most highly expressed in the cortex, hippocampus, and striatum during late prenatal and early postnatal development, suggesting they could play a particularly important role in mediating the response to early life stressors (Feng et al., 2005; 2007; 2010; Miller et al., 2012; Simmons et al., 2012; Matrisciano et al., 2013; Simmons et al., 2013).

DNA is typically bundled into nucleosomes of chromatin that are 'wrapped' around histone proteins. The post-translational modification of histone tails, by methylation, acetylation, or phosphorylation can alter the position of DNA binding points (interactions), and how tightly DNA is bound to the histone (binding affinity), can alter the accessibility of the DNA to transcription factors and polymerases, thus altering the DNA's ability to be transcribed and expressed. While histone acetylation appears to 'loosen' the chromatin, allowing for increased transcription, histone deacetylation and methylation may more tightly bind the DNA, and prevent access by transcription factors and enzymes, though relationships between methylation and transcription may be somewhat more complex (reviewed in Mcgowan and Roth, 2015).

It is important to note here that these epigenetic changes can again only occur within the context of the specific genome and alleles being acted upon. It is possible that only certain genes may be vulnerable to epigenetic modification during development, and that a person's genetic complement may alter their allelic sensitivity to methylation. For example, allelic differences between individuals can alter sensitivity to dietary folate and other methyl-donors in epigenetic programming, with some groups and individuals seeing greater risk reduction with multivitamin supplementation than others (Etheredge et al., 2012). Nevertheless, epigenetic changes may provide an important molecular mechanism by which maternal stress, and the early environment, can impact fetal programming of later neuropsychiatric disease susceptibility.

1.3.1. Epigenetic Changes Linked to Neuropsychiatric Disorders

Clinically, epigenetic changes have been linked to both depression and response to antidepressant treatments (reviewed in Menke and Binder, 2014). In postmortem

studies, both DNMT1 and DNMT3a are highly expressed in GABAergic neurons in patients with schizophrenia or bipolar disorder (Veldic et al., 2005; 2007; Poulter et al., 2008; Zhubi et al., 2009; Peter and Akbarian, 2011; Zhu et al., 2011; Murphy et al., 2012; Kofink et al., 2013; Dong et al., 2014; Halene et al., 2014), while gene polymorphisms in the DNMT3a or DNMT3B gene, as well as altered patterns of global methylation, have also been associated with suicide attempts in psychiatric patients (Maciag et al., 2010; Murphy et al., 2012). Peripheral markers of methylation (in white blood cells, saliva, or cheek swabs), also show changes in patterns that are correlated with depression. Decreased levels of DNMT1 and increased levels of DNMT3b have been found in the white blood cells of patients currently experiencing a depressive episode, but not those in remission (Higuchi et al., 2011). Lower levels of DNMT3b prior to significant stress are associated with a greater risk of post-traumatic stress disorder (PTSD) and more severe PTSD symptoms, while higher post-stress levels of DNMT1 were found in PTSD cases compared to controls (Sipahi et al., 2014). Again using peripheral blood levels, individuals with a life history of depression exhibit different methylation patterns in a number of genes associated with brain development, serotonin function, and immune responses, all of which have been associated with depression (Uddin et al., 2011).

Interestingly, some animal studies have found that antidepressants or mood stabilizers can decrease levels of DNMT1 and DNMT3a in specific brain regions, and some effects of antidepressants can be mimicked by blocking methylation (Melas et al., 2012; Zimmermann et al., 2012). Antidepressants have also been found to reduce hyper-methylation of brain-derived neurotrophic factor (BDNF), an important nerve growth factor associated with both depression and antidepressant treatment (Duclot and Kabbaj,

2015). In a rodent model, rats bred for high levels of anxiety and depressive behaviour showed significant decreases in the levels of DNMT1 in the basolateral and central amygdala on PND7, but these differences were absent by PND14, suggesting an important role of transient early life methylation changes in the development of anxiety behaviours (Simmons et al., 2012).

1.3.2. Epigenetic Changes Linked to the Early Environment

Several studies have linked epigenetic changes to prenatal stress, in both human and animal models (Mueller and Bale, 2008; Peña et al., 2013; Provençal and Binder, 2015). Changes in methylation of the promoter region of the glucocorticoid receptor gene (NR3C1) have been found in umbilical cord blood of offspring of mothers exposed to prenatal depression or extreme stress situations, including partner violence (Oberlander et al., 2008; Radtke et al., 2011). Some recent studies of peripheral blood cells have also noted significant differences in DNA methylation markers in patients with a history of childhood abuse (Mehta et al., 2013; Suderman et al., 2014). One study using buccal cheek cells reported that exposure to early life stress significantly altered DNA methylation at a number of CpG sites in adolescence, and these changes were sex-specific (Essex et al., 2013). However, the majority of human studies of early life stress rely on peripheral markers, which may not always correlate with brain changes, though one postmortem study has reported significant changes in methylation in NR3C1 in people who committed suicide who also experienced child abuse, but not in those suicides who did not experience abuse (Mcgowan et al., 2009).

In rodent models of adult chronic stress, sex-specific effects on CRF in the male and female PVN and CeA are associated with altered patterns of CpG methylation in the

CRF gene (Sterrenburg et al., 2011). Chronic social defeat has been linked to increased levels of DNMT3a levels in the NAc (Laplant et al., 2010), as well as to increases in histone acetylation in the PFC and hippocampus, and these changes appear to correlate with behavioural changes following chronic stress (Kenworthy et al., 2014). Prenatal restraint stress early in gestation has been linked to reduced CRF gene methylation in the hippocampus and amygdala of male mice (Mueller and Bale, 2008). Interestingly, prenatal exposure to a predator (considered an ethologically relevant psychological stressor) during late gestation alters methylation of the BDNF promoter, resulting in decreased levels of BDNF, in the hippocampus of female, but not male offspring (St-Cyr and Mcgowan, 2015), suggesting both sex and stressor type could contribute to differences in epigenetic modifications following prenatal stress. As discussed in **Section 1.1.3**, the placenta normally prevents exposure to high levels of circulating GCs in the fetus by acting as a barrier to GCs, but prenatal stress can weaken this barrier (Gheorghe et al., 2010; St-Pierre et al., 2015). Recent research shows that methylation changes in the 11βHSD2 gene promoter may provide the mechanism for these changes, increasing the levels of GCs that can reach the developing fetus (Peña et al., 2013), providing another facet of development by which epigenetic changes could contribute to the effects of prenatal stress.

A number of epigenetic changes are also associated with postnatal stressors or interventions (reviewed in Jawahar et al., 2015). Maternal deprivation results in decreased HDAC in the NAc, an effect that can be prevented or rescued by adult treatment with the antidepressant imipramine (Réus et al., 2013b), while variations in maternal care have been extensively shown to alter transcription regulation of adult GR in

the hippocampus (Weaver et al., 2004; Szyf et al., 2005; Weaver et al., 2005). Beyond that, recent research has found that maternal care induces a broad range of other epigenetic changes in the adult brain (Mcgowan et al., 2011). Interestingly, postnatal 'abuse' significantly increased methylation of the BDNF gene promoters in the PFC (Roth et al., 2009), in addition to causing sex- and age-specific changes in methylation of BDNF and reelin genes from infancy to adulthood in the mPFC (Blaze et al., 2013), another brain region associated with both stress responding and neuropsychiatric disorders. Conversely, epigenetic changes may also underlie some of the beneficial effects of environmental enrichment, as adult environmental enrichment alters aspects of histone acetylation in the hippocampus and cortex following neurodegeneration (Fischer et al., 2007), and BDNF promoter methylation in the hippocampus (Kuzumaki et al., 2011). Early life social enrichment altered aspects of histone acetylation of BDNF promoters in the hippocampus (Branchi et al., 2011). Despite the evidence for epigenetic alterations in response to environmental change in adulthood, few studies have examined the role epigenetic changes play after enrichment early in life and, in particular, the potential for sex differences in its effects; thus, a more complete investigation of these processes is needed.

Taken together, the literature suggests that epigenetic changes may be a key mechanism linking prenatal stress, the early environment, and sex differences in the later development of neuropsychiatric disorders. Despite the evidence that early life events cause long-term changes in epigenetic markers that are found in adulthood, characterization of when these disrupted processes are first found during development is in its infancy, even though it is an important component of understanding the

mechanisms and trajectories underlying later mental health disorders. Further, some of these underlying epigenetic changes may themselves be transient, setting off a cascade of epigenetic changes or modifications that dynamically alter the trajectory of development, rather than cause a single fixed change to the epigenome (Crudo et al., 2013). Thus, a better understanding of the role of epigenetic changes in the development of anxiety/depressive disorders, as well as potential relevance of sex and different time points in targets for interventions, requires more research on time points in early life, in both 'normal' development, and following early life stressors.

1.4. Sexual Differentiation and Early Life Development

As described in the previous sections, there are significant sex differences in vulnerability to a number of neuropsychiatric disorders, including anxiety and depression, as well as differences in symptoms, progression, and severity of these mental health disorders (reviewed in Altemus et al., 2014; Bangasser and Valentino, 2014; Loke et al., 2015). In addition, there are a number of sex differences in stress responding, as well as differences in the effects of stress on both the brain and behaviour across development (reviewed in Pacak et al., 1998; Laviola and Macrì, 2013; Graignic-Philippe et al., 2014) that may contribute to vulnerability to these disorders (reviewed in Hodes, 2013). These sex differences in the brain are determined by a number of genetic, hormonal, and environmental or experience factors, and are partially established during the initial 'wiring' of the sexually dimorphic brain by permanent organizational effects of hormones, as well as by later acute activation by circulating gonadal hormones.

1.4.1. The Role of Gonadal Hormones in Sexual Differentiation

Male and female brains are first differentiated, in part, during a sensitive developmental period by exposure to hormones. In rodent models, the perinatal testes release two surges of gonadal testosterone, one around GD18, and another within a few hours of birth on PND0. In humans, fetal sex hormones first emerge in gestational week 7, peaking between 12-18 weeks of pregnancy and then dropping off, surging again at birth once maternal inhibitory factors are no longer in play (reviewed in Altemus et al., 2014). These surges are controlled by the expression of the sex-determining region Y (SRY) gene on the Y chromosome, which regulates initial testis development. This process has been well characterized in rodent models, where, in some regions of the brain, testosterone is converted into estradiol by the P-450 aromatase enzyme, aromatase, to act on estrogen receptors (ER) ERα and ERβ, or converted by 5α reductase to an androgen metabolite, dihydrotestosterone (DHT), which binds to androgen receptors (AR; reviewed in Mccarthy, 2010). Together, early life hormones are thought to play a key role in organization of the male and female brain, by a combination of masculinizing and defeminizing the male brain, and feminizing the female brain. Masculinization is defined as a series of active processes initiated by the gonadal hormones during the sensitive period that result in 'typical' adult male behaviours, while feminization is considered the 'default' pathway which occurs in the absence of masculinizing hormones and results in 'typical' adult female behaviours. Defeminization is the active removal of the ability to express 'typical' female behaviours (reviewed in Mccarthy, 2010).

Both androgens and estrogens act within the brain by binding to ARs and ERs. The more well known effects are through binding to intracellular ARs or ERs that then

translocate to the nucleus to act as nuclear transcription factors to alter gene expression (either assisting or inhibiting expression, depending on a number of other cofactors, corepressors, and immediate early genes). In contrast, both ARs and ERs also have a number of rapid, non-genomic actions, that are controlled by receptors binding to membrane bound G-proteins, protein kinases, sex-hormone binding globulin receptors, voltage and ligand-gated ion channels, or transporters (reviewed in Michels and Hoppe, 2008; Wilson et al., 2011; Handa et al., 2012). Though an extensive review of the mechanisms involved in sexual differentiation is beyond the scope of this chapter, it is important to note that the actions of androgens and estrogens within the brain are modulated by the steroidogenesis enzymes, as well as ARs and ERs, all of which are expressed in sexually dimorphic spatiotemporal patterns in different brain regions, highlighting the importance of both enzymes and receptors in the effects of these androgen and estrogen hormones in different brain areas (Konkle and Mccarthy, 2011; Wilson et al., 2011; Tsai et al., 2015).

These genomic and non-genomic actions can trigger a number of changes in the brain, impacting processes such as neuronal migration, neurogenesis, differentiation, cell death, and synaptogenesis, as well as a number of mediators and neurotransmitters, including endocannabinoids, prostaglandins, glutamate, GABA, and vasopressin (reviewed in Mccarthy, 2010). Through these processes, early life hormones contribute to the development of sex differences in the size, structure, and phenotype of many brain regions, as well as connections between and within these regions. Notably, this highly dynamic and spatiotemporally dimorphic process provides an important pathway through which risk or resilience to mental health disorders can be established in ‘typical’

development, as well as a target for disruptions by early life environmental cues to act in a sex, time, and region specific manner. Though the majority of research into the process of sexual differentiation of the brain has focused on neural and behavioural changes linked to reproduction, an increasing body of evidence suggests that the process of sexual differentiation, and its sensitivity to disruptions by the early environment, likely play a key role in the biological sex differences in vulnerability to mental health disorders.

1.4.2. Early Life Gonadal Hormones and Anxiety/Depressive Behaviours

Clinically, sex differences in prenatal testosterone exposure may contribute to some aspects of anxiety or depressive disorders. In mid-gestation, lower circulating levels of (amniotic) testosterone are associated with both a lower response to reward, and an increased negative response bias in an fMRI task in pre-pubertal boys (Lombardo et al., 2012a) - markers which have also been previously associated with later depressive disorders (Lopez-Duran et al., 2012a; 2012b; Das et al., 2013; Hopkins et al., 2013; Leyfer et al., 2013). Variations in amniotic testosterone have also been correlated with sex differences in gray matter volume in some brain regions (Lombardo et al., 2012b), and these levels of circulating testosterone can also be disrupted by environmental factors (Caramaschi et al., 2012). Patients with Klinefelter syndrome, a chromosomal disorder in which men carry an extra X chromosome (XXY), demonstrate hypogonadism, and although it is difficult to separate the hormonal and chromosomal effects in these individuals, research suggests that they are at a greater risk of a number of neuropsychiatric disorders, including depression, anxiety, attention-deficit hyperactivity disorders and schizophrenia, along with a number of changes in neural grey matter, suggesting a possible link between low levels of testosterone and these disorders,

although much work remains to be done in characterizing prenatal hormones in these individuals (reviewed in Høst et al., 2014). More directly, prenatal exposure to endocrine disruptors such as diethylstilbestrol (DES) or bisphenol A (BPA) may increase adult risk of anxiety or depression (O'Reilly et al., 2010), and concentrations of BPA in prenatal urine have been shown to correlate with increased symptoms of anxiety and depression in boys at 7 years old (Harley et al., 2013). These findings mirror several animal studies where BPA increases both anxiety and depressive behaviours (Fujimoto et al., 2006; Tian et al., 2010; Jašarević et al., 2013; Kundakovic et al., 2013).

In rodent models, prenatal exposure to excessive androgens increases anxious behaviours and correspondingly down-regulates androgen receptors in the amygdala of male rats, without altering adult circulating sex steroid levels. Further, these changes can be prevented by either prenatal flutamide (anti-androgen) or tamoxifen (an estrogen receptor modulator), suggesting an important role for both organizational androgens and estrogens in rodent anxiety behaviours (Hu et al., 2015). Exposure to flutamide in the perinatal period has also been shown to increase depressive behaviours in juvenile male rats, prior to puberty, emphasizing a specific role of androgens in preventing depressive behaviours early in life (Zhang et al., 2010a). Similarly, pre-pubertal castration alters adult response to novelty in male rats, another important indicator of early life hormones on adult anxiety/depressive behaviours (Brown et al., 2015). In a marble-burying task in mice, where lower rates of burying are typically present in females (interpreted as a more passive/anxious response to the stress of the task) perinatal testosterone exposure resulted in a more male-typical ‘active’ coping response (increased burying rate) in adulthood, suggesting a role for pre-pubertal testosterone in the lower levels of anxiety behaviours

typically seen in males on this task (Goel and Bale, 2008). In addition to behavioural changes, organizational androgens have also been found to play a role in immune system development, and exposure to perinatal flutamide has been shown to increase the pro-inflammatory response to a bacterial lipopolysaccharide (LPS) challenge in adult males (Ongaro et al., 2011). Interestingly, several studies have now linked increased inflammation and immune dysfunction to depression vulnerability, and anhedonia in particular (Anisman, 2009; Audet et al., 2014; Kim et al., 2015).

In addition to these behavioural and immune system changes, organizational hormones play a key role in the development of a number of brain regions described in **Section 1.2** that are linked to anxiety/depressive disorders. In the PFC, AR mRNA increases transiently in males in the perinatal period (Mogi et al., 2015), and prenatal exposure to flutamide decreases markers of dendritic growth in this region (Pallarés et al., 2014). In the hippocampus, flutamide treatment altered aspects of neurogenesis and synaptogenesis (Zhang et al., 2010a), while perinatal BPA exposure altered NMDA receptor density (Xu et al., 2010). In the amygdala, prenatal BPA exposure also altered the expression of ERs (Cao et al., 2013), while prenatal exposure to high levels of testosterone decreased ARs, but increased serotoninergic and GABAergic receptor expression in the adult female amygdala, and increased female anxiety behaviours (Hu et al., 2015). Further, both perinatal DHT and estradiol play a role in the amygdala-dependent development of juvenile social play behaviours, and these changes appear to be epigenetically mediated (Auger et al., 2011; Jessen and Auger, 2011; Kolodkin and Auger, 2011).

1.4.3. Early Life Gonadal Hormones and the Ontogeny of Stress Responding

Beyond the evidence suggesting a role for organizational androgens and estrogens in anxiety and depressive behaviours, early life hormones also alter aspects of HPA axis development, an important mediator of the effects of stress in both early life and adulthood. In a rat model, treatment during the last week of gestation and the early postnatal period (from GD13-PND20), with either flutamide or an aromatase inhibitor (1,4,6-androstatriene-3,17-dione; ATD) increased CRF mRNA and decreased GR mRNA in the PVN of adult male rats, and flutamide treatment also increased AVP. These treatments increased circulating levels of corticosterone in adulthood, both basally and in response to stress (Seale et al., 2005b). Neonatal castration or exposure to testosterone also altered stress responding, having opposite effects on the corticosterone response to stress in adulthood, as well as fos response to stress in the PVN, and these effects could be prevented by neonatal, but not adult, testosterone replacement (McCormick and Mahoney, 1999; Bingham and Viau, 2008). Interestingly, the effects of organizational hormones appear to go beyond acute response to stress, as male pups exposed to either ATD or flutamide from PND0-PND21 failed to show the typical habituation in plasma corticosterone levels following repeated stress in adulthood (Bingham et al., 2012). Further, prenatal stressors have themselves been shown to disrupt surges of perinatal testosterone (Kapoor and Matthews, 2011; Walf and Frye, 2012; Pallarés et al., 2013a), and this hormonal disruption could contribute to the development of sex differences in some of the neural and behavioural changes observed following early life stress (Barrett et al., 2013).

Organizational effects of sex hormones on HPA function have been investigated in females as well, but the results have been less consistent, with fewer long-term changes. Neonatal treatment with estradiol resulted in an adult pattern of CRH mRNA, AVP mRNA and GR in the PVN that was intermediate between that found in males and females, and these effects were not normalized by adult estradiol treatment (Patchev et al., 1995). However, another study found that neonatal testosterone altered HPA function in adulthood, but these effects were normalized when the groups were gonadectomized and given estradiol as adults, suggesting the effects were the result of altered circulating estradiol levels in adulthood caused by the perinatal treatment (Seale et al., 2005a).

As was described in **Section 1.1.3**, the early postnatal period is a time of low levels of circulating corticosterone and decreased HPA activity known as the SHRP. As discussed earlier, certain types of stressors appear to activate the HPA axis during this period and several studies have reported no sex differences in the stress response at this time (reviewed in McCormick and Mathews, 2007). However, one study has reported a stronger corticosterone response in females than males on PND3 in response to an endotoxin, and these sex differences could be prevented or reversed by gonadectomy (Shanks et al., 1994), while another found a sex differences in the adrenal sensitivity on PND14 that could be prevented by either neonatal castration in males or postnatal testosterone treatment in females (Yoshimura et al., 2003), suggesting that the response to certain types of stressors during this period may be sexually dimorphic.

To summarize, the literature suggests a complex and varied role for early life organizational hormones in the development of behavioural changes related to anxiety/depression, as well as in programming the stress response and creating sexual

dimorphisms in regions associated with these behaviours. However, there is still a relative paucity of research directly investigating the role of these early life hormones in shaping sex differences in the brain and behaviour beyond reproduction. Even less is understood about how or when these changes arise developmentally, what mechanisms are involved, or how they may dynamically alter these brain regions early in life, particularly in ways that may predict the later development of adult disorders. Thus, a more complete picture of the role of organizational hormones in the development of anxiety/depressive disorders, as well as potential relevance of sex and early time points as targets for interventions, requires a more in depth study of early developmental time points in both sexes during 'typical' development and following alterations to organizational hormone levels.

1.4.4. Early Life Gonadal Hormones and Epigenetic Changes

As described in **Section 1.3**, epigenetic mechanisms are emerging as an important driver of both the long-term programming of the brain following early life stress, and the vulnerability to neuropsychiatric disorders. Intriguingly, recent research has suggested that epigenetic changes in histone modifications and DNA methylation are also a key component of normal hormone-driven sexual differentiation of reproductive behaviours (reviewed in Mccarthy and Nugent, 2013). In the sexually dimorphic nucleus of the preoptic area (SDN-POA), exposure to perinatal estradiol decreased activity of DNMTs, resulting in decreased methylation that appeared to allow for activation of more 'masculinizing' genes. Further, preventing the action of DNMT3a in the early postnatal period was found to masculinize some aspects of female adult sexual behaviour, as well as brain regions related to reproduction including the SDPOA (Nugent et al., 2015).

Though still a fairly new area of research, recent work by Auger and colleagues has demonstrated that the expression of DNMT3a in the postnatal amygdala is a key factor in organizing sex differences that typically correlate with juvenile play behaviour, with female rats expressing higher levels of DNMT3a in the amygdala on PND1 relative to males. Further, organizational hormones appear to be involved in manifesting this difference, as DHT or estradiol treatment decreased levels of DNMT3a to more male-typical levels (Kolodkin and Auger, 2011). Acetylation and methylation of histone H3 also appears to be sexually dimorphic in early life, from E18-PND6, in brain regions including the hippocampus and cortex, and prenatal testosterone treatment has been found to masculinize H3 acetylation levels, but not methylation levels, in these brain regions (Tsai et al., 2009). The role of epigenetic changes downstream of early life hormones has also been noted in research on endocrine disruption, where BPA treatment altered levels of DNMT1 and DNMT3a in the hippocampus, prefrontal cortex and hypothalamus, and these changes correlated with changes in ER gene expression in these regions (Kundakovic et al., 2013). DNMT3a is known to be involved in sexual differentiation (Mccarthy and Nugent, 2015; Nugent et al., 2015) and, as discussed previously, has been linked to some anxiety and depressive changes in the brain and behaviour. DNMT3a is also particularly highly expressed in the late prenatal and early postnatal period (Feng et al., 2005), a period known to be important both for sexual differentiation and the long-term programming of stress effects. Thus, it appears to be an important contender for involvement in the hormone-driven sexual differentiation of non-reproductive brain regions, particularly those associated with stress and depression. As mentioned previously, a better understanding of the pathways by which these epigenetic

changes contribute to anxiety or depression vulnerability is vital to our understanding of the etiology of these mood disorders, and may provide new options for potential treatment targets and earlier time points for identification of disorders. Given the potential importance of sex differences in these epigenetic mechanisms, more research is needed into how and when these differences arrive in both sexes. Further, given sex differences in the prevalence, symptoms, and time course of these disorders, the study of the epigenetics of sexual differentiation may itself provide an important natural model for vulnerability or resilience in these disorders.

1.5. GABA as a Mechanism Linking Stress, Sex and Depression

Global studies of DNMT methylation levels and HDAC activity do not tell us about what specific neuronal properties or neurotransmitter systems are being altered by these epigenetic changes during development. Changes to the gamma-amino–butyric-acid (GABA) neurotransmitter system are one possible link among early life stress, sex differences, and vulnerability to anxiety or depressive disorders. GABA is the primary mediator of fast synaptic inhibition in the adult brain and it regulates neural function in a number of important ways. GABA is produced from glutamate by the glutamic acid decarboxylase (GAD) enzyme, which has two isoforms – GAD67, 67 kDa, and GAD65, 65kDa, which are encoded by the GAD1 and GAD2 gene respectively. The two isoforms appear to have different expression patterns in the cell, as well as potentially different functions in early life, where GAD67 is the predominant form expressed in early prenatal development, while GAD65 is expressed later on. GAD65 is produced primarily at the axon terminal synapse for rapid release, and is often present in an inactive state (not bound to pyridoxal 5'phophate – PLP), only being activated in response to demand for

synaptic GABA release. GAD67 is critical for prenatal development, is found in the cytoplasm, and appears to be responsible for cellular interactions, tonic inhibition, and cellular metabolism, and is responsible for the majority of GABA synthesis in the brain, though it may also play a direct role in inhibitory neurotransmission (reviewed in Pehrson and Sanchez, 2015). Across early development, GABAergic neurons are also differentiating into subtypes of GABAergic cells that express different calcium-binding proteins, including parvalbumin, calretinin or somatostatin (reviewed in Cancedda et al., 2007; Müller et al., 2015).

GABA acts on ionotropic GABA-A and GABA-C receptors, as well as metabotropic GABA-B receptors. GABA-A receptors have several configurations with different properties and different sensitivities to drugs or agonists, based on the particular combination of subunits, $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ, π, and $\rho_{1\text{-}3}$, making up a pentamer, with the most common combination including two α, two β and one γ subunit. The most plentiful subtype configuration in adulthood includes two α_1, two β_2, and one γ_2 subunit, which acts to mediate fast, phasic inhibition. Early in development, there are more α_2, α_3 and α_5 subunits and extra-synaptic GABA-A receptors that allow for longer duration tonic currents that are crucial for cell migration, proliferation, and synapse formation. These are gradually replaced by α_1 across the postnatal period as brain regions mature and these processes slow down (Ehrlich et al., 2013; Le Magueresse and Monyer, 2013; Deidda et al., 2014). The precise timing of the shift in subunit composition likely plays a role in development, and may be important in creating a vulnerability to later anxiety or depressive disorders (reviewed in Smith and Rudolph, 2012; Rudolph and Mohler, 2014). For example, a transgenic knockout of α_1 receptors early in development alters

conditioned fear learning (Wiltgen, 2009), while knockdown in adulthood does not alter anxiety behaviour (Heldt and Ressler, 2010).

In addition to the changes in receptor subunits, and the differentiation into different types of interneurons, GABA also matures from an excitatory to inhibitory neurotransmitter early in development (reviewed in Ben-Ari et al., 2012). The action of GABA at GABA-A receptors is excitatory in prenatal and early postnatal development, and acts as the primary driver of early neuronal migration, growth, maturation, and synapse formation (Ganguly et al., 2001). The developmental switch from excitation to inhibition is driven by a change in the intracellular concentration of chloride, the primary ion mediating GABA-A currents. Early on, the sodium-potassium-chloride co-transporter (NKCC1) is highly expressed, and serves to transport chloride into the cell, creating a high intracellular concentration that results in a positive shift in membrane potential when GABA binds to GABA-A receptors (i.e., an excitatory response). Across postnatal development, the expression of the potassium-chloride co-transporter 2 (KCC2) increases, which acts to transport chloride from the cell, creating the more mature chloride gradient that renders GABA action at GABA-A receptors inhibitory. Early in life, excitatory GABA has been shown to result in calcium influx, and as a result, to regulate synapse formation and neuronal growth, including the formation of excitatory glutamatergic synapses (reviewed in Ben-Ari et al., 2012). Indeed, blocking NKCC1 while GABA is typically depolarizing can decrease dendritic length and transmission at excitatory synapses (Wang and Kriegstein, 2011), while prematurely switching GABA from excitatory to inhibitory (by expressing KCC2 early) alters dendritic morphology (Cancedda et al., 2007). Interestingly, the increased expression of KCC2 and the timing

of the subsequent shift from excitation to inhibition are influenced by both sex and the early environment (reviewed in Galanopoulou, 2008a; Nuñez and Mccarthy, 2008; Ben-Ari et al., 2012; Le Magueresse and Monyer, 2013).

Notably, the maturation of these various components of the GABAergic system are highly dependent on brain region, and, as discussed below, are sensitive to the environment in early life and are often sexually dimorphic, making developmental GABA an interesting candidate linking sex, stress, and anxiety/depressive disorders. The upcoming sections will describe some of the existing research linking GABA to anxiety/depression and briefly outline some of the studies looking at GABA in early development, particularly as it relates to early life stress and sexual differentiation.

1.5.1. GABA Changes Linked to Neuropsychiatric Disorders

There is growing evidence that a GABAergic deficit could be an important factor in the development of depression (Luscher and Shen, 2011). GABergic neurons are pervasive, with projections in all of the brain regions described in **Section 1.2** that are linked to anxiety/depressive disorders, including the nucleus accumbens, amygdala, prefrontal cortex, and hypothalamus, as well as the hippocampus (reviewed in McKlveen et al., 2015). GABA is also involved in regulating the release of other neurotransmitter systems, including modulating those linked to depression, including dopamine, norepinephrine, and serotonin (reviewed in Croarkin et al., 2011). It also directly influences the PVN to help control the HPA axis response to stress (reviewed in de Souza and Franci, 2013), making it an important candidate mechanism for the development of depression. In several postmortem studies, reduced GAD67 has been found in the prefrontal cortex (Rajkowska et al., 2007; Sequeira et al., 2009; Maciag et al., 2010; Gos

et al., 2012; Seney et al., 2013), and PVN (Gao et al., 2013), as well as in the cerebrospinal fluid (CSF) and plasma of depressed patients (reviewed in Gao and Bao, 2011). Despite reports of decreased GAD67, no studies to date have reported differences in levels of GAD65 between depressed patients and healthy controls (reviewed in Pehrson and Sanchez, 2015). Markers of GABAergic neurons, including somatostatin and corticostatin are also down-regulated in the postmortem amygdala of women with depression (Guilloux et al., 2011), and several recent studies have noted changes in GABA receptor subtypes in the PFC and amygdala following suicide and major depressive disorders (Merali et al., 2004; Poulter et al., 2008; Sequeira et al., 2009; Poulter et al., 2010). Imaging studies have revealed decreased cortical GABA levels in depressed patients compared to controls (Sanacora et al., 2004; Hasler et al., 2010; Klumpers et al., 2010; Levinson et al., 2010, reviewed in Croarkin et al., 2011), and these decreases may be partially recovered by antidepressant treatment (Sanacora et al., 2002). Notably, one study has found decreased cortical GABA levels even in patients currently in remission, suggesting that decreased GABA could be an underlying trait marker for disease vulnerability, rather than a requirement for an active depressed state (Bhagwagar et al., 2008). This means that GABAergic changes may be an important developmental marker for the disease. Indeed, a recent study has shown significant differences in excitatory and inhibitory responses to transcranial magnetic stimulation (TMS) in children and adolescents with clinical depression compared to controls (Lewis et al., 2016). The role of GABA in depression is further supported in some treatment studies, as treatment with antidepressants normalizes aspects of GABA in the brain and plasma in patients (Sanacora et al., 2002; Taylor et al., 2006; Gören et al., 2007). Importantly,

however, several other studies do not show this decrease in post-mortem GABA levels, nor are there consistent effects of GABAergic drugs on mood, and an excellent recent review of the literature suggests the relationship may be more "nuanced and complex" than a straightforward reduction across all brain regions and patients, highlighting a need for more research into the nature and direction of inhibitory GABA effects in major depressive disorders (Pehrson and Sanchez, 2015).

The clinical literature also suggests a potential role for GABA in the development of anxiety disorders. The major anxiolytic drugs, benzodiazepines, are believed to target ligands at a particular GABA-A receptor subtype that is highly concentrated in the BLA (Sieghart, 1992; Olsen and Sieghart, 2009), and levels of GABAergic markers in the BLA are correlated with anxiety levels in rodent models (Barbalho et al., 2009; Flores-Gracia et al., 2010; Tzanoulinou et al., 2014). Some clinical studies suggest that *in vivo* GABA binding is decreased in the cortex in anxiety disorders (Nikolaus et al., 2010), with decreased benzodiazepine receptor binding in the PFC linked to PTSD (Bremner et al., 2000), and in the frontal cortex with panic disorder (Hasler et al., 2008). Allopregnanolone, a positive modulator at GABA-A receptors, is decreased in the CSF of women with PTSD, and these women have an altered sensitivity to GABA-A receptor drugs (Rasmusson et al., 2006; Möller et al., 2014). Interestingly, reduced levels of allopregnanolone in the CSF and plasma have also been reported in some clinical studies of depression, and up- or down-regulating the neurosteroid in animal models have also been associated with changes in anxiety/depressive behaviours (reviewed in Schüle et al., 2014), suggesting it could be an important moderator of co-morbid anxiety and depression.

Results from the use of several different animal models provide support for the idea that there is link between disruptions to GABA and anxiety/depressive disorders. A temporary knockout of the GABA-A receptor γ2 subunit in the early postnatal period disrupted HPA axis function, increased anxiety behaviour on the elevated plus maze, and altered sucrose consumption anhedonia and forced swim test 'despair' in adulthood (Shen et al., 2010; 2012). Injecting a GABA-B receptor antagonist prenatally altered anxiety/depressive behaviours in adult male and female rats, and blunted the HPA axis response to stress (Stratton et al., 2014). In a genetic model of depression, Flinders-Sensitive Line (FSL), researchers have also found that, compared to control rats, there is a significant increase in levels of the chloride co-transporter KCC2 in several brain regions, which suggests that increased GABA transmission could relate to the increased depressive behaviours in this model (Matrisciano et al., 2010). Though less work has focused on early life models of anxiety, the basolateral amygdala is a critical site for the anxiolytic actions of benzodiazepines in rodent models, and several studies report correlations between GABA disruptions in the amygdala and increased anxiety (reviewed in Ehrlich and Rainnie, 2015; Maeng and Milad, 2015). Interestingly, activation of GABA-A receptors with an agonist in the early (PND3-5) postnatal period significantly disrupts adult HPA axis activity and increases adult anxiety and depressive behaviours in male mice, while later (PND14-16) disruptions caused a milder change in the HPA axis and anxiety behaviours without altering depressive behaviours, suggesting that early life GABA plays a time-sensitive role in the development of brain circuits involved in later anxiety and depressive disorders, as well as stress responding (Salari et al., 2015).

Taken together, these results suggest that disruptions to inhibitory GABA play a key role in neuropsychiatric disorders, but do not offer any clues as to when or how these differences first arise. As discussed briefly in the previous section, GABA is highly plastic and sensitive to the external environment in early life, and, as we will examine below, appears to be critically disrupted by early life stressors.

1.5.2. GABA Changes Linked to the Early Environment

As discussed in **Section 1.1.2,** stress in early life is an important risk factor for developing later neuropsychiatric disorders. As described in the previous section, GABA is an interesting potential target for early life insults, as a number of parameters of the GABA system are established during this same time frame (reviewed in Levitt et al., 2004). GABA is also known to play a key role in brain maturation (Ben-Ari, 2002; Ben-Ari et al., 2007; 2012) and the timing of the switch from excitatory to inhibitory GABA is both sexually dimorphic and sensitive to environmental disruptions (Galanopoulou, 2007; He et al., 2010; Matrisciano et al., 2010; Cossart, 2011; Hyde et al., 2011; Lee et al., 2011; Ehrlich et al., 2013; Murguía-Castillo et al., 2013), providing an interesting link between prenatal stress and sex in anxiety/depression.

As discussed earlier in the chapter, one of the most well-established effects of prenatal stress is to alter HPA axis function in adulthood; changes in the stress response and HPA axis rhythms are often reported in anxiety and depressive disorders (Suzuki et al., 2013; Walker et al., 2013). The final common pathway of the glucocorticoid response is mediated by CRH neurons of the PVN, which receives substantial inputs from local inhibitory GABAergic inputs, and integrates responses from regions of the limbic forebrain, including the mPFC, DMH, BNST, and amygdala (reviewed in Herman et al.,

2004; Cullinan et al., 2008), regions that are also linked to depressive disorders (reviewed in Goldstein et al., 2014). Indeed, disruptions to typical embryonic development of the GABA system are associated with sex-specific changes to the PVN formation, as well as the HPA axis response to stress in adulthood (Stratton et al., 2014), while mice lacking a subtype of the GABA-B receptor show increased sensitivity to stress-induced anhedonia and show social avoidance (O'Leary et al., 2014). Further, recent research has demonstrated that, in the PVN, glucocorticoid receptors co-localized with a subset of GABAergic neurons, demonstrating a direct role of GABA in mediating the GR feedback on the HPA axis response (de Souza and Franci, 2013).

Results from a number of animal studies support the idea that there is a link between prenatal stress and disrupted GABA; prenatal exposure to maternal immune activation (MIA) alters GABA content and subunit levels, disrupts later working memory (Richetto et al., 2013; 2014) and alters GABA in a regionally specific fashion, decreasing GAD67 protein levels in the adult NAc, but increasing them in the mPFC (Li et al., 2015b). Prenatal injections of the synthetic corticosterone hormone dexamethasone (DEX) decrease calretinin expression in the amygdala of adult female, but not male offspring, while prenatal restraint stress decreases GAD in the frontal cortex and hippocampus of adult female, but not male, rats (Van den Hove et al., 2013). Interestingly, baseline levels of GABA in the brain may interact with these prenatal stressors to produce changes in early life. One recent study has found that mice with lower levels of GAD67 are more sensitive to the effects of prenatal and maternal stress than those with more GAD67 available (Uchida et al., 2011), highlighting the importance of both underlying genotype and stress in creating later GABAergic disruptions.

Similarly, prenatal stress decreased proliferation rates of parvalbumin cells in the PFC and hippocampus in mice with reduced expression of GAD67, but not in wild type mice (Uchida et al., 2014). Prenatal stress also altered aspects of GABAergic neuron migration and altered the expression of genes involved in determining the differentiation or fate of the interneurons in mice with deficits in prenatal GAD67 (Stevens et al., 2012).

In addition to prenatal stressors, changes in the early postnatal period can also affect development of the GABAergic system. Maternal separation has been found to alter tonic GABAergic currents and levels of GABA in the hippocampus (Feng et al., 2014), decrease the density of parvalbumin neurons in the PFC (Leussis et al., 2012) and decreases GABA-A receptor α subunits in a sex and region specific manner in pubertal and post-pubertal animals (León Rodríguez and Dueñas, 2013). GAD67 mRNA levels in the hippocampus and amygdala also demonstrate dynamic regulation in response to stressors during the stress hyporesponsive period (Dent et al., 2007), suggesting an important role for GABA in responding to the environment during this time frame. Maternal care variations (in particular maternal licking and grooming), also seem to alter GAD levels, with increased licking and grooming resulting in decreased methylation of hippocampal GAD67, subsequently increasing GAD67 levels in the hippocampal GABA system (Caldji et al., 2003; Zhang et al., 2010b, reviewed in Champagne, 2013). Increased concentrations of GABA in the PFC of both juvenile male and female offspring have also been reported following early life environmental enrichment (Connors et al., 2015).

Notably, the majority of the studies described here using either prenatal or postnatal interventions examined the long-term (usually adult) effects of these

interventions on GABA, and there are very few papers examining the developmental expression of these effects (reviewed in Fine et al., 2014). It is therefore unclear whether the effects of prenatal stress on adult GABA discussed above are the result of direct mediation of prenatal GABA, or are indirect through other neurotransmitter systems. Further, it is possible that initial GABAergic changes in early development could be in the opposite direction of some of these adult effects, with compensatory mechanisms or changes in other aspects of the excitatory/inhibitory balance resulting in the final disruptions to GABA markers reported above. More research into the developmental changes in GABA is needed before we can interpret these changes beyond recognizing that there are disruptions to adult GABAergic circuitry caused by prenatal stress.

1.5.3. GABA Changes Linked to Sexual Differentiation

In addition to interactions between early life stress and GABA, some of which are sexually dimorphic, many aspects of typical GABAergic development display sex differences, and are regulated in part by the organizational effects of early life gonadal hormones. The results of one study showed that males have higher levels of GABA in the hypothalamus and hippocampus early in postnatal life (PND1), but not later on in postnatal development (Davis et al., 1999). The timing of the switch from excitatory to inhibitory GABA is also sexually dimorphic, occurring at different points during early development in males and females, though the precise timing of the shift is region dependent. For example, the change in NKCC1/KCC2 ratio that underlies the switch from excitatory to inhibitory GABA occurs at different times in the male and female hippocampus and entorhinal cortex, usually shifting by the end of the first postnatal week in females, but remaining depolarizing until the second week in males (Nuñez and

McCarthy, 2007; Murguía-Castillo et al., 2013). In the substantia nigra, the shift appears around PND10 in females, but around PND17 in males (Galanopoulou et al., 2003; Galanopoulou, 2007), and levels of KCC2 are significantly higher on PND5 in females than males in the hypothalamus (Auger et al., 2001; Perrot-Sinal et al., 2007) and these changes appear to be related to early life estradiol and testosterone (Nuñez and McCarthy, 2007; Perrot-Sinal et al., 2007; Murguía-Castillo et al., 2013 reviewed in Galanopoulou, 2008a). The magnitude and duration of the calcium influx that accompanies depolarization as a result of activating GABA-A receptors also appears to be sexually dimorphic in the early hippocampus, is correlated with increased expression of the γ_2 subunit of the GABA-A receptor, and is altered by exposure to early life androgens (Nuñez and Mccarthy, 2008). Similarly, early life levels of GAD, the rate limiting enzyme for GABA synthesis, are sexually dimorphic in some brain regions, including the CA1 region of the hippocampus, the BNST, and the hypothalamus, and these differences are also linked to early gonadal hormone surges (Davis et al., 1996b; 1999; Perrot-Sinal et al., 2001; Polston et al., 2004; Zhou et al., 2005). Given the important role for depolarizing GABA in neuronal maturation, migration, and synapse formation discussed previously, this body of research points to an important role for sex differences in developmental GABA in shaping the brain in a sexually dimorphic fashion.

Notably, much of the existing research on the role of sexually dimorphic GABA in the development of the brain has focused on the hypothalamus, hippocampus, or substantia nigra (reviewed in Mccarthy and Nugent, 2013; 2015). As discussed in **Section 1.4**, there is evidence that early organizational hormones can have long-term programming effects on brain regions linked to anxiety/depressive disorders, including

the PFC (Pallarés et al., 2014; Mogi et al., 2015) and amygdala (Auger and Olesen, 2009; Jessen and Auger, 2011; Kolodkin and Auger, 2011; Cao et al., 2013; Hu et al., 2015). Though the role of GABA in the development of these sex differences has not been well studied, some evidence suggests that sex differences in the number of GABAergic cells (Stefanova, 1998), as well as the density of GABA-B receptors (Hu et al., 2015), in the adult amygdala might be the result of organizational androgens. Further, as described previously, evidence suggests GABA is a key system through which early life stress might disrupt development in brain regions linked to anxiety/depression, including the nucleus accumbens (Li et al., 2014), amygdala (Laloux et al., 2012; Zuloaga et al., 2012a; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015) and PFC (Leussis et al., 2012; Matrisciano et al., 2013; Holland et al., 2014; Uchida et al., 2014; Van den Hove et al., 2014; Labouesse et al., 2015), and some of these effects are themselves sexually dimorphic (Van den Hove et al., 2013; Holland et al., 2014; Marron Fernandez de Velasco et al., 2015). Together, then, this research highlights a potential role of early life sex differences, and thus (potentially) organizational hormones, in the developmental trajectory of GABA in these anxiety/depression associated brain regions.

1.5.4. GABA Changes Linked to the Epigenetic Regulation

As described in **Section 1.3**, changes in the epigenome are likely a key mechanism driving the long-term alterations in the brain induced by prenatal stress, are a significant player in the process of sexual differentiation, and are a source of vulnerability for the later development of neuropsychiatric disorders. However, studies of overall changes in DNMT methylation levels and histone modifications do not provide information on the particular neurotransmitter systems or receptors being epigenetically

modified, and researchers must begin the correlate these changes in overall methylation with changes in other markers of interest, as well as directly examining the changes in methylation patterns of specific genes, to better isolate epigenetic effects within the brain and provide more specific targets for treatment interventions.

The GABAergic system, in addition to being involved in sex differences, early life stress, and depression, also appears to be an important target of early life epigenetic modifications. There is converging evidence that the decreased expression of GAD in schizophrenic patients, and patients with major depression, is associated with increased methylation of the GAD1 gene (reviewed in Dalton et al., 2014; Halene et al., 2014). DNMT1 and DNMT3a in particular are highly expressed in GABAergic neurons in patients with neuropsychiatric disorders (Guidotti et al., 2005; Veldic et al., 2007; Poulter et al., 2008; Thompson et al., 2009; Zhubi et al., 2009; Maciag et al., 2010; Croarkin et al., 2011; Luscher and Shen, 2011; Peter and Akbarian, 2011; Guidotti et al., 2013; Kofink et al., 2013). In a mouse model where GAD67 positive cells also express green fluorescent protein (GFP), DNMT1 and DNMT3a appear to co-localize with GAD67-ir neurons (more so than glutamatergic neurons or glia) in a number of regions, including the hippocampus, amygdala, striatum, piriform and motor cortices (Kadriu et al., 2012). Further, overexpressing DNMT1 has been linked to both increased anxiety and decreased GAD67 in the rodent amygdala (Zimmermann et al., 2012), while some of the effects of maternal care have also been linked to hypermethylation of the GAD1 promoter in low licking and grooming dams (Zhang et al., 2010b). Interestingly, a recent study demonstrated that perinatal exposure to BPA resulted in increased anxiety behaviours in female rats, which correlated with both overexpression of DNMT1 and reduced GAD67

mRNA in the BLA, providing evidence of links between GABA, epigenetic changes, organizational hormones and anxiety/depressive behaviours (Zhou et al., 2013), though little research has focused on the intersection of these different areas of research.

Taken together, this section describes evidence for GABAergic changes in early development as an important contributor to the development of anxiety and depressive disorders, and highlights potential links between GABA, early life stress, sexual differentiation, and epigenetic changes that could provide the groundwork for establishing GABA's role in underlying vulnerabilities to these disorders. However, as illustrated throughout the section, there is a relative scarcity of research focusing on the developmental trajectory of these effects (reviewed in Fine et al., 2014), particularly including both male and female subjects. A better understanding of if and when these changes are present in early life would improve both our understanding of the stages involved in developing these disorders, and potentially allow us to identify vulnerable points earlier in life to target interventions, perhaps even before clinical disorders manifest fully.

1.6. – Chapter Conceptual Summary

Throughout this chapter I have outlined several key points when considering the development and use of animal models of anxiety and depressive disorders: 1) early life stress is well established as a risk factor for neurodevelopmental disorders – but more research is needed on when and how underlying neural changes first arise. A better understanding of how these early stressors disrupt the developmental trajectory of both brain and behaviour could play a key role in understanding how and when vulnerabilities to neuropsychiatric disorders emerge, 2) epigenetic changes are implicated as a key

underlying mechanism in several neurodevelopmental disorders, including anxiety and depression, and understanding how and when these changes first emerge early in development could improve our understanding of the pathophysiology of anxiety and depression 3) sex differences in both the response to early life stress, and in typical brain development, likely contribute to emergence of anxiety/depressive disorders, as well as vulnerability to later risk factors, 4) understanding how early life stress might influence epigenetic and neural development in sex-specific ways requires a better understanding of the course of typical sexual differentiation of the brain, and 5) GABAergic changes are a prime candidate to mediate some of the sex-specific effects of early life stress, as they have been linked to both epigenetic changes in neurodevelopmental disorders, as well as sexual differentiation in the brain.

The remainder of this thesis describes several experiments in which I attempt to investigate some of these gaps in our understanding of the developmental trajectory of vulnerability to anxiety/depressive disorders. In **Chapter 2**, I present the results of an investigation using a novel, ethologically relevant model of prenatal stress (exposure to predator; PPS), along with a postnatal enhanced housing condition (EHC), on the developmental trajectory of anxiety/depressive disorders. The results represent a novel examination of behavioural changes in both juvenile and adult animals, looking at how and when changes first emerge in both males and females, and if these early behavioural tests can provide insight into later adult vulnerabilities. In **Chapter 3**, I begin to investigate how this model of PPS and EHC might be interfering with early brain development in both males and females, by examining epigenetic changes in DNMT3a at the same time point as our early behavioural testing. In **Chapter 4**, I examine how the

process of sexual differentiation, and in particular circulating androgen levels, contribute to sex differences in DNMT3a-ir at the same time point as our behavioural and PPS/EHC paradigms. In **Chapter 5**, I examine the role of the GABAergic system as a candidate neurotransmitter being disrupted by our PPS/EHC model, by examining GAD67-ir density at this same early behavioural time point. In **Chapter 6**, I will then investigate the role of gonadal androgens in establishing the developmental patterns of GAD67-ir in these juvenile animals. Finally, **Chapter 7** includes a general discussion of findings, integrating the results of Chapters 2-6, as well as a consideration of study limitations, and some suggestions for future research directions.

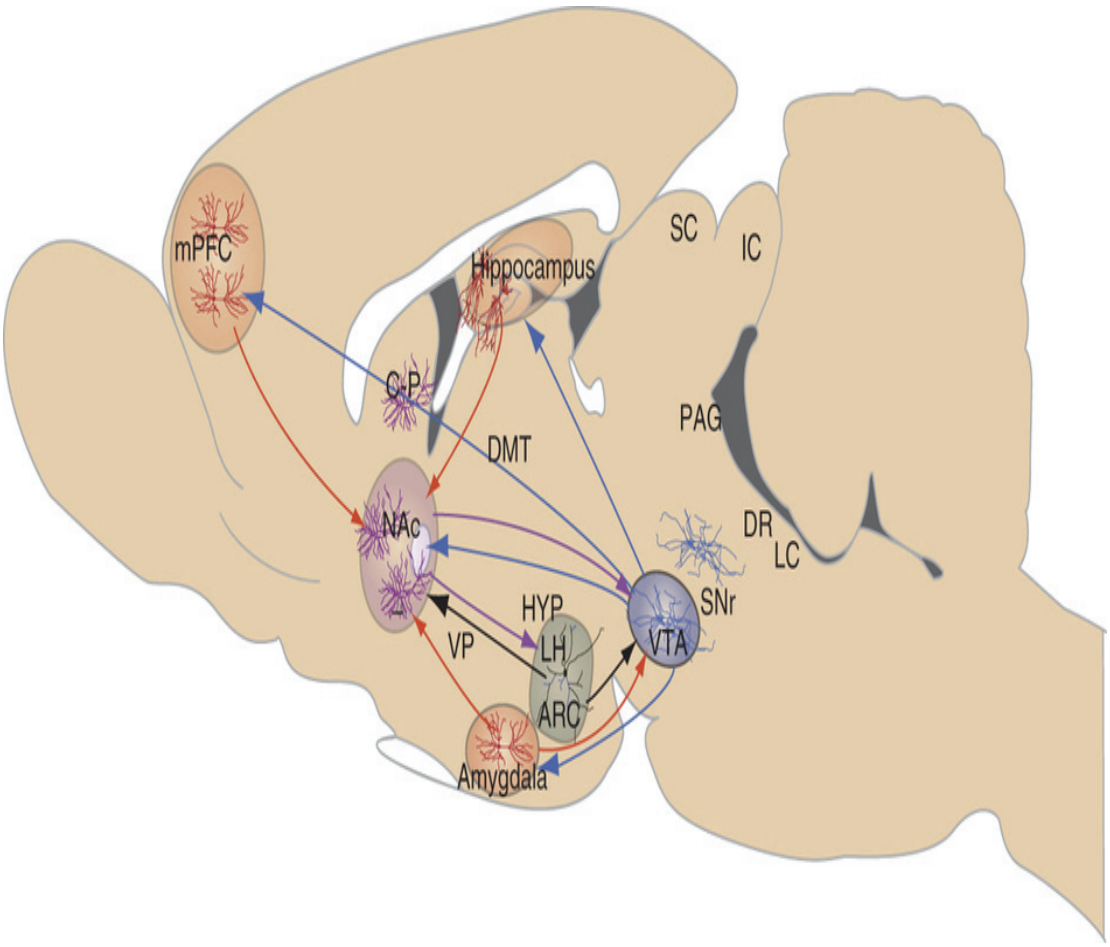

FIGURE 1.1. MAJOR BRAIN STRUCTURES INVOLVED IN ANIMAL AND HUMAN MODELS OF DEPRESSION.

Depicted are the major brain structures in mood-related circuits that are altered by stress in animal models of depression or implicated in human depression. The red solid lines represent excitatory glutamatergic afferents to NAc from mPFC, amygdala and hippocampus, and glutamatergic innervation of VTA by amygdala. GABAergic afferents (purple) are inhibitory circuits and include connections from NAc to VTA and hypothalamus. Dopamine neurons (blue solid lines) project from VTA to a range of limbic targets, including NAc, mPFC, amygdala and hippocampus. Peptidergic pathways through which the hypothalamus (for example, ARC, arcuate nucleus, and LH, lateral hypothalamus) alters neurotransmission in NAc and VTA are shown in solid black lines. Each structure contains specialized neuronal cell types thought to regulate stress responses, including resilience. These cell types, color-coded to reflect the transmitter signal they convey, include amygdala, PFC and hippocampal glutamatergic neurons (red), GABAergic NAc medium spiny neurons (purple), hypothalamic peptidergic neurons (black), and VTA dopaminergic neurons (blue). CP, caudate-putamen; DMT, dorsomedial thalamus; SC, superior colliculus; IC, inferior colliculus; VP, ventral pallidum; SNr, substantia nigra; PAG, periaqueductal gray; DR, dorsal raphe; LC, locus coeruleus. Figure and Caption are adapted with permission from the Nature Publishing Group (See Appendix 1; Russo et al, 2012).

CHAPTER 2 – DEVELOPMENTAL EXPRESSION OF ANXIETY AND DEPRESSIVE BEHAVIOURS AFTER PRENATAL PREDATOR EXPOSURE AND EARLY LIFE HOMECAGE ENHANCEMENT

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2.1. Introduction

Childhood mental health is increasingly being recognized as a key public health issue. In children, prevalence rates are between 0.3-7.8% for depression (Belfer, 2008; Lima et al., 2013; Lewis et al., 2014) and between 2.2-9% for anxiety disorders (Rapee et al., 2009; Leyfer et al., 2013). In addition to the negative consequences of anxiety and depressive symptoms on a child's quality of life, these disorders can have long-term impacts on a child's physical, psychosocial, and cognitive development. Children with high levels of anxiety and depressive symptoms are also at an increased risk of developing later mental health problems and co-morbid disorders, with worse long-term outcomes than those diagnosed as adults (Kessler et al., 2001; Roza et al., 2003; Mason et al., 2004; Rapee et al., 2009; Korczak et al., 2013). Childhood mood and behavioural problems share many clinical symptoms with adult mood disorders, which suggests that mood disorders in children may be an early manifestation of some of the same underlying neuropsychiatric issues found later in life. Further, childhood symptoms of anxiety or depression, as well as associated neurophysiological changes can predict later adult vulnerability to many neuropsychiatric disorders, even at subclinical levels (Mason et al., 2004; Leyfer et al., 2013; Pagliaccio et al., 2015). Given the high economic burden and increasing prevalence of these neuropsychiatric problems, there is significant interest in identifying risk factors and markers of anxiety and depression in early life, both to enhance our understanding of the developmental trajectory of these diseases, and to target early interventions (Côté et al., 2009; Loth et al., 2014).

While the prevalence of depression in children is not negligible, rates of diagnosis increase markedly across adolescence, from 4-8% to up to 25% in early adulthood

(Kessler et al., 2001). Perhaps because of this higher post-pubertal incidence of clinical anxiety and depressive disorders, much of the existing animal behavioral research has focused on modeling anxiety and depressive behaviors in adult animals, even when examining the impacts of early life risk factors such as prenatal stress (reviewed in Weinstock, 2008; Buynitsky and Mostofsky, 2009). However, the human literature suggests that many physiological and behavioural effects of risk factors such as early life stress or maternal anxiety can already be seen in very young children, and these changes may predict later vulnerability to anxiety/depressive disorders (reviewed in Garber, 2006; Shankman et al., 2009; Kovacs and Lopez-Duran, 2010). Though many rodent tests for anxiety- and depressive-like behaviours were developed for adults, they also appear to have some validity in juvenile animals. Rat pups as young as day 21 have shown a response to antidepressants in the Forced Swim Test (FST) of behavioural despair (Reed et al., 2008; 2009), as well as increased FST immobility in response to acute restraint stress (Bernal-Morales et al., 2009), and increased anxiety-like behavior in the Open Field Test (OFT; (Laloux et al., 2012). In genetic models of depression (Flinders Sensitive Line – FSL, and Wistar Kyoto – WKY) recent research has found that pre-pubertal rats already demonstrated some behaviours associated with both childhood and adult depression in humans (saccharin anhedonia, FST 'despair', and co-morbid anxiety in the OFT). Interestingly, these animals also demonstrate some more childhood-specific symptoms including disrupted social play behavior and a failure to respond to some types of antidepressant treatments, along with age-specific changes in the HPA and monoamine systems (Malkesman and Weller, 2009), which supporting the use of these behavioural tests in pre-pubertal animals to model 'childhood' depression. There is a critical need to

examine this relationship between early life risk factors such as prenatal stress (described below) and juvenile anxiety/depressive behaviours, in order to help determine when and how these behavioural changes first manifest. Further, as with the clinical literature, our understanding of the interactions between underlying risk factors and the developmental trajectory of anxiety/depressive disorders could be greatly improved by longitudinal research in these animal models, to examine how these juvenile behaviours might predict later adult symptoms (Stevens and Vaccarino, 2015).

Stress in early life is well established as a risk factor for the development of adult anxiety/depressive disorders (reviewed in Duman, 2010; Schmidt, 2011; Morley-Fletcher et al., 2013; Glover, 2014; Pizzagalli, 2014). Prenatal stress exerts long-term effects on both physiology and behavior – disrupting hypothalamic-pituitary- adrenal axis (HPA) function, altering aspects of brain development, and contributing to vulnerability to depressive and anxiety disorders in both human (Hines et al., 2002; Van den Bergh et al., 2005; 2008; Davis and Sandman, 2010; Kleinhaus et al., 2013) and animal models (reviewed in Goel and Bale, 2009; Glover et al., 2010). In animal models, the type of stressor used (along with the timing and duration of exposure) is a key factor in both the maternal response to the stressor, and the subsequent brain and behavioural changes in the offspring (Kaiser and Sachser, 2005; Neumann et al., 2005; Tazumi et al., 2005; Léonhardt et al., 2007; Davis and Sandman, 2010; Mychasiuk et al., 2011b). In rats and mice, prenatal stress studies most often use repeated restraint under bright lights (reviewed in Darnaudéry and Maccari, 2008; Weinstock, 2008; Buynitsky and Mostofsky, 2009) to induce a physiological response in the pregnant dam. Though many of the stressors used in rodents (including foot shock, restraint) have a strong physical

component, in human pregnancy the stressors experienced are more likely to be predominantly psychological (Abe et al., 2007). One recent study has demonstrated that a prenatal physical swim stressor and a psychological 'bystander stressor' (observing another animal in distress), had unique and sex-specific effects on later adult cognitive and motor behaviours in rats (Nazeri et al., 2015). Together, this suggests that in order to better model the human prenatal stress experience, and to expand our understanding of the myriad of ways in which stressors might affect development, more 'psychological' prenatal stress paradigms need to be characterized in animal models of anxiety/depressive behaviours, particularly in early life.

Exposure to predator odor is an ethologically relevant, unconditioned, psychological stressor in rodents (Apfelbach et al., 2005; Takahashi et al., 2008). Exposure to predator odor has been shown to alter dopamine metabolism in the amygdala, and to activate brain regions including the medial amygdala, the bed nucleus of the stria terminalis (BNST), the medial hypothalamus structures, and the periaqueductal gray (PAG) – regions involved in processing stressful/fearful responses (Wiedenmayer et al., 2000; Oliveira et al., 2012). Predator odor exposure also elicits physiological and behavioral responses, particularly increased defensive and anxiety behaviours, that persist well beyond the stressor paradigm (Blanchard et al., 1998; Morrow et al., 2000; McGregor et al., 2004; Muñoz-Abellán et al., 2008; Mashoodh et al., 2009; Muñoz-Abellán et al., 2009; Butler et al., 2011). Further, unlike other repeated stress paradigms, repeated predator exposure does not induce complete habituation of either the HPA axis response or behavioural response to the stressor (Figueiredo et al., 2003; Mashoodh et al., 2009). Acute exposure to a predator odour during late gestation

has also been found to increase anxiety-behaviours in the postpartum dam (Toumi et al., 2013), and to decrease postpartum maternal care (Patin et al., 2002), while postnatal exposure to a predator odor has been found to increase or decrease maternal care depending on the timing of exposure and other environmental cues (McLeod et al., 2007; Kenny et al., 2014). Prenatal predator stress might therefore produce a unique maternal stress experience that is psychological in nature, and subsequently alter the fetal and postnatal environment in ways that are distinct from the effects of more physical stress paradigms, creating a unique model of the developmental trajectory of anxiety and depressive behaviours.

To date, scant research exists on the effects of prenatal predator stress, with existing studies focusing primarily on effects on seizures (Saboory et al., 2011), where prenatal predator stress increased juvenile seizures compared to prenatal restraint stress (Ahmadzadeh et al., 2011), though effects are sex and dam dependent (Korgan et al., 2014). We are only aware of one study examining the effects of prenatal predator stress on adult behaviours, but the research is promising; prenatal predator odour increased offspring anxious/defensive behaviours, and resulted in female-specific changes in the corticosterone response to threat as well as in the hippocampus and amygdala (St-Cyr and Mcgowan, 2015). Interestingly, postnatal exposure to predator odor also altered anxiety behaviours in adult offspring in a sex-specific manner (increasing open field anxiety behaviours in males, and decreasing them in females; (Mashoodh et al., 2009), suggesting that early life predator stressors may have timing and sex-specific effects on later vulnerability to adult anxiety/depressive behaviours, though again there is very little research on when these changes first arise during development.

In addition to the effects of prenatal stress, the juvenile brain remains exquisitely sensitive to postnatal interventions, which could modulate the effects of prenatal programming, and potentially rescue or repair some of the effects of prenatal stress (reviewed in Connors et al., 2015; Crofton et al., 2015). Environmental enrichment rearing (enrichment in the period immediately after weaning and into adolescence) may be effective in preventing or reversing some of the effects of early life stressors on adolescent learning and normal social play (Francis et al., 2002; Morley Fletcher et al., 2003), as well as adult anxiety (Baldini et al., 2013). Earlier environmental enrichment, during the immediate postnatal period, may also prevent depressive behaviours in adult females (Welberg et al., 2006). Communal rearing, another naturalistic model of environmental enrichment, effects both the brain and behavior in very young animals; reducing ultrasonic vocalizations (USV; a measure of anxiety/emotionality) in response to maternal absence on PND8, as well as reducing adult anxiety and depressive behaviours in the FST and elevated plus maze (Cirulli et al., 2010), though these effects may be sex-dependent (D'Andrea et al., 2010). In our laboratory, a postnatal enhanced housing condition (EHC) has been shown to reduce the likelihood, and severity, of febrile convulsions in PND15 juveniles following repeated prenatal predator stress (Korgan et al., 2014). This suggests that our EHC, as an early environmental intervention, may already have important effects on the juvenile brain and behaviour, and could play an important role in mediating or 'rescuing' any effects of prenatal predator stress on juvenile anxiety/depressive behaviours.

Along with the substantial increase in depressive disorders during adolescence, in clinical studies a significant sex difference in prevalence (females higher than males)

emerges after puberty, suggesting an important interaction between early brain development, sex, and peri-pubertal plasticity in the emergence of adult clinical anxiety and depression. Interestingly, in rodent models of early life stress a greater vulnerability to depressive behaviours is often reported in adult females (Zagron and Weinstock, 2006; Toufexis, 2007 reviewed in Weinstock, 2007), though some studies suggest anxiety and learning may be more disrupted in males (Yaka et al., 2007) and depend on the timing of the stressor (Mueller and Bale, 2007; 2008; reviewed in Weinstock, 2011). Given the importance of early life programming on organizing sex differences (reviewed in Lenz et al., 2012; Mccarthy et al., 2012; Seney et al., 2012), sex-specific changes following prenatal stress may result in sex differences in anxious or depressive behaviours, even in juvenile animals. These sex differences could then serve as markers for, or even contributing factors to, later vulnerability to adolescent and adult mood disorders. Sex differences in behavioural responses to prenatal stress (greater anxiety in females) have been reported as early as PND30 (a peripubertal time point; Baker et al., 2008; Schroeder et al., 2012), though prenatal stress increased juvenile FST immobility in both sexes (Zhang et al., 2013). The effects of early life environmental enrichment also appear to be sex-specific. Prenatal and postnatal environmental enrichment has been shown to prevent some of the effects of later stressors on the HPA axis in adult females, with no effects in males (Welberg et al., 2006). In our laboratory, postnatal EHC has been found to preferentially decrease febrile convulsions in juvenile females compared to males (Korgan et al., 2014), again suggesting that some of these effects may already be manifesting in observable behavioural changes in very young animals.

Taken together, the literature suggests that interactions between the early

environment (prenatal and early postnatal), sex, and aspects of the stress response in contributing to mood disorders in adults have been well characterized. However, there are significant gaps in our understanding of how these factors contribute to the development or manifestation of anxious or depressive behaviours in juveniles (particularly using both male and female subjects), and how those are then related to the adult phenotype. Moreover, the research performed using an ethologically relevant prenatal stressor is very scarce in both juveniles and adults, as are experiments in which there are attempts to 'rescue' the effects of prenatal stress with early interventions such as early life enrichment. In the present study, I exposed pregnant Long-Evans rats to repeated predator exposure during the last week of gestation, and evaluated the impact on both corticosterone levels in dams and juveniles, and anxiety/depressive behaviours in juvenile rats. I also assessed the potential preventative or rescue effects of an enhanced postnatal housing condition on these behaviours. I then followed these same animals over time, observing their peri-pubertal responses to a social interaction task believed to be related to juvenile depression (Morley Fletcher et al., 2003; Malkesman and Weller, 2009), and on to adulthood, where we observed whether early corticosterone or behavioural changes could predict adult responses on tests of anxiety and depressive behaviours.

2.2. Methods

2.2.1. Animals and breeding

Male (225-250g) and Female (175-200g) Long-Evans hooded rats were obtained from Charles River Canada (St. Constant, Quebec), and housed in same-sex pairs while allowed to acclimate to the colony room for at least one week before breeding. After acclimation, females' estrous cycles were monitored daily with vaginal smears. Females

in proestrus were paired overnight with a sexually experienced Long–Evans male from the colony. Successful mating was confirmed the next morning by the presence of sperm in vaginal smears, and this was considered gestational day 0 (GD0). Mated females were then removed from males' cages and rehoused with one other recently inseminated female until GD12. At that point, females were singly housed and remained singly housed with their litters throughout the remainder of the experiment. With the exception of enhanced home cages (EHC) used from PND0 to PND22 for 10 mothers and their offspring, rats were housed in standard colony rooms in Plexiglas cages (22 x 21 x 44 cm) with wire mesh lids, pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a 15 cm long black polyvinyl-carbonate (PVC) tube for enrichment. The colony rooms were maintained at 21±2°C, and on a reverse 12:12 hour light:dark cycle with lights off at 07:00h. Rats had *ad libitum* access to food (Purina Lab Chow) and tap water.

Unless otherwise specified, all experimental manipulations were performed during the rats' active phase (dark cycle) under red light. Housing conditions and all experimental procedures were pre-approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and were in accordance with the guidelines stipulated by the Canadian Council on Animal Care. An effort was made to use the minimum number of animals required for statistical comparison to minimize pain and suffering in experimental subjects.

2.2.2. Prenatal and postnatal environmental manipulations

Figure 2.1 depicts the experimental timeline for perinatal manipulations. Starting on GD13, pregnant females were randomly assigned to one of two prenatal conditions;

naïve control (NC) or prenatal predator stress (PPS), and then to one of two postnatal conditions upon giving birth, either standard cage (SC) or enhanced homecage (EHC), with groups as follows; i) NC/SC (n=5), ii) NC/EHC (n=5), iii) PPS/SC (n=5), and iv) PPS/EHC (n=5). From GD13-GD20, dams in the PPS groups were exposed 3 times daily (30 min/exposure; 08:30h, 11:30h, 14:30h) to a predator threat. Dams were transported to one of the three cat colony rooms in our department and their cages placed on to the floor to allow the cats to investigate. All exposures took place under red light. During each exposure period, dams designated as NC were similarly placed in clean cages but were maintained in the colony room under red light for the duration of the 30 min. On the first, third, sixth, and eighth day of the prenatal stressing (GD13, 15, 18, 20), fecal samples were collected from both PPS and NC dams twice daily over two-hour windows beginning at 08:00h (AM sample) and 14:00h (PM sample), and the fecal boli were labeled and stored at -20°C for future assay. As circulating glucocorticoids take approximately 6 hours to be maximally excreted in the feces (Cavigelli, 2005), fecal boli deposited during the 2 hr collection period from 14:00 - 16:00h represented circulating levels around the time of the first exposure of the day from 08:30 – 09:00h. Likewise, samples deposited during the 2 hr collection from 08:00 to 10:00h represented circulating levels during the prior light phase, and were taken to represent more basal GC levels. After each exposure period, PPS and NC dams were returned to their home cages and the colony room. All dams were checked daily for pups starting on GD20 at the start of the dark cycle. On the day of delivery, designated Postnatal Day 0 (PND0), litters were sexed, and all pups were counted and weighed. This was performed quickly to minimize pup handling. Dams and pups designated for exposure to an EHC were transferred to

EHC cages at this time and remained in them until weaning (PND22). Enhanced home cages were approximately 2.5x the volume of standard cages divided into an upper section, where food and water was available *ad libitum* (as well as a PVC tube for further enrichment) and a lower section simulating a burrow (see Figure 2.2). Dams and pups designated as SC controls were transferred to a fresh standard cage (described in section 2.1) on PND0. All cages (both SC and EHC) were changed weekly. All pups were weaned on PND22, and transferred to fresh standard cages to be housed in same-sex sibling pairs (described previously). Offspring continued to be maintained a reversed 12:12 hour light:dark cycle and had *ad libitum* access to food and water, with the exception of during the sucrose preference test.

2.2.3. Fecal glucocorticoid extraction and assay

Fecal samples collected from dams during pregnancy on GD 13, 15, 18 and 20 were used to estimate circulating levels of GCs in both NC and PPS females. Samples were collected during both AM (8:00-10:00h) and PM (14:00-16:00h) periods to attempt to capture both baseline and stress levels of GCs (Touma et al., 2004; Cavigelli, 2005). During extraction, samples were allowed to thaw to room temperature and the number of fecal boli and the wet mass (g) of each sample was recorded. Samples were flash-frozen in liquid nitrogen and manually homogenized using a mortar and pestle, after which 0.1 g of sample was combined with 1 ml of 80% methanol, vortexed for 30 min, and centrifuged for 15 min at 2500 × g. The resulting supernatant was collected and stored at −80◦C. Corticosterone metabolites were quantified from supernatant, diluted 1:40 in duplicate using Assay Design's Corticosterone correlate EIA Kit (Assay Designs, Michigan, USA) according to the manufacturer's instructions. Intra- and inter-assay

coefficients of variation for this kit ranged between 6.6% and 8.0% and 7.8% and 13.1%, respectively. In addition to corticosterone, the major metabolites tetrahydrocorticosterone and 11-dehydrocorticosterone are also present in rat fecal samples (Thanos et al., 2009). This assay kit had the following cross-reactivities with steroid compounds of importance to fecal sampling: corticosterone 100%; 11-deoxycorticosterone 28.6%; tetrahydrocorticosterone 0.18%.

2.2.4. PND15 Cardiac Puncture Blood Collection and Plasma Corticosterone Assay

On PND15, one male and one female pup collected from dams exposed to the prenatal/postnatal paradigm, but not to behavioural testing, were sacrificed for plasma collection to examine circulating glucocorticoid levels, and then perfused for brain collection for separate experiments (Korgan et al., 2014). After deeply anesthesizing animals with a lethal dose of Euthanyl (sodium pentobarbital), a 5ml syringe with a 23G needle was inserted into the heart and a blood sample was drawn. Blood was collected in 1.5 ml Microtainer plasma separator tube containing lithium heparin (Becton Dickinson, United States), and kept on ice until samples were centrifuged at 6000 x g for two minutes at 4 °C. Plasma was collected and frozen at -80 °C until assay. Levels of corticosterone were determined using Assay Design's Corticosterone correlate EIA^{TM} Kit (Assay Designs, Michigan, USA), according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation for this kit range between 6.6 - 8.0% and 7.8 - 13.1%, respectively, and the lower limit of detection is 32 pg/ml.

2.2.5. Behavioural testing

Two male and two female pups were randomly selected from each litter for behavioural testing on PND15, resulting in n's of 8-10 pups of each sex in each treatment

group. In order to minimize carryover effects, the sequence of juvenile behavioural tests was chosen such that the least aversive tests were performed first (Perrot-Sinal et al., 2004), and all animals underwent behavioural testing in the same order. The timeline for behavioural testing was as follows: Juvenile Open Field Test (OFT-J) on PND15, Juvenile Forced Swim Pre-Test (FSPT-J) on PND16, Juvenile Forced Swim Test (FST-J) on PND17, a Social Interaction test on PND30 (SI), a Sucrose Preference Test (SPT) on PND75, an Adult Open Field Test (OFT-A) on PND76, and an Adult Forced Swim Test (FST-A) on PND77. Although there may have been lasting effects of some of these potentially stressful behavioural tests on subsequent behavioural measures, these effects would have been consistent across all treatment groups. All testing was performed early in the animals' dark cycle, between 09:00h and 12:00h. Each behavioural test was video-recorded for later analysis.

Juvenile open field test. On PND15, all pups underwent OFT-J. The open field apparatus consisted of a black 72 cm (l) x 72 cm (w) x 30 cm (h) Plexiglas box with no lid. The floor was divided into 16 squares of equal dimensions using brightly coloured tape to facilitate quantification of locomotion. Prior to testing, home cages were transported to the testing room, placed on an electric heating pad at a low setting, and left undisturbed for a ten-minute habituation period under dim light. For each test, pups were placed in the center of the novel arena, and allowed to explore the arena for 5 minutes under bright light. This open field protocol has been used successfully for assessing effects of prenatal stress at PND10-15 (Mychasiuk et al., 2011c), amphetamines at PND15 (Laviola et al., 2004a), and anxiety behaviors in response to acute stress at PND22 rats (Bernal-Morales et al., 2009), with similar dimensions used for juvenile rats in another study (Zhang et al.,

2010a). Before each test, and between pup tests, the arena was wiped clean using a 1% bleach cleaning solution and dried with paper towel. After each test, the pups' fur was coloured using non-toxic permanent ink markers to facilitate identification for future testing. Each OFT session was video recorded by a camera mounted on a tripod positioned on a table such that the camera extended approximately 2 m above the arena and was angled to allow optimal visualization of behaviours. Videos were scored for measures of the animals' activity levels and exploratory/investigative tendencies: *line cross frequency*, defined as crossing a line with both forepaws; *rearing time (s)*, defined as time spent standing on hind paws with body stretched upwards, unsupported by forepaws; time spent *grooming (s)*, defined as time spent in rapid cleaning movements of the forepaws towards the face or body (Abe et al., 2007). Measures of emotionality (anxiety/fear) behavior were also obtained by analyzing *freezing time (s),* defined as time spent in total immobility*,* and *time spent in the center (s)*, defined as time spent with all four paws in one of the four central squares of the arena, compared to outer squares of the arena (an area of relative safety; Choleris et al., 2001).

Juvenile forced swim test. On PND17, pups underwent a 10-minute juvenile forced swim test (FST-J) according to a modified version of the original protocol proposed by Porsolt et al. (1978). The test was conducted in a 22 cm diameter x 35 cm high clear plastic 5000 ml beaker filled with approximately 3500 ml of water such that the rats were neither able to reach the floor or rim of the container. Water temperature was maintained at 26±1°C, and the cylinder refilled with fresh water before every test. Twenty-four hours prior to the testing session, rats were subjected to a fifteen-minute forced swim pre-test (FSPT) performed under the same conditions as the juvenile FST. The FSPT facilitates

development of immobility during subsequent tests and has been shown to increase the sensitivity of the test to the effects of antidepressants (Detke et al., 1995). Upon removal from the water after completion of both the FSPT-J and FST-J, pups were gently dried with paper towel and placed in a drying station lined with paper towel and illuminated by a bright, warm lamp for ten minutes. The pups were re-coloured with permanent marker prior to being returned to their cages. Throughout the pre-test and test sessions, pups that were not currently undergoing testing or drying were kept in standard cages placed on electric heating pads at low temperature settings. A camera was mounted above the swimming chamber on a tripod, and both the juvenile FSPT and juvenile FST were recorded for later analysis. The durations of three behaviours were measured based upon criteria established by Reed et al. (2008) specifically for juvenile rats (PND21): *swimming (s)*, defined as an almost horizontal body position, in which the rat undergoes horizontal displacement across the water's surface by paddling both its hind and forepaws; *climbing (s)*, defined as a somewhat vertical body position in which the rat's hind legs kick vigorously below it while its forelimbs engage in upward directed motions against the cylinder's walls; *time spent immobile (s)*, defined as having little (enough to keep the rat from drowning) to no body movements, such that the rats maintain a non-traversing position, and *latency to first immobility*, defined as the latency to first exhibit immobility behaviour (described above) for 2 seconds or more. Duration of immobility and latency to immobility are thought to reflect behavioural despair (Porsolt et al., 1977), and were used to infer depression-related behaviour.

Social interaction test. The social interaction test paradigm was adapted from the methods published by Morley-Fletcher et al. (2003). On PND29, same-sex sibling cage

mates were separated into individual standard cages in the colony room, and remained separated for the twenty-four hours prior to the test period to increase social engagement during the test (Cirulli et al., 1996). Social interaction tests were conducted on PND30 in a quiet testing room under red-light illumination. This date was chosen because social play is observed to peak in rats between PND16 and PND35 (Vanderschuren et al., 1997). During the test, same-sex sibling pairs were reunited in a fresh standard cage, and left undisturbed for 20 minutes, with their behaviours video-recorded by a camera mounted on a tripod next to the test cage. Behaviours were scored according to the protocol presented by Morley-Fletcher and colleagues (2003), whereby the durations of three social and two non-social behaviours were recorded. Social behaviours included: *rough-and-tumble play*, defined as pouncing (subject lunges toward its partner with its forepaws extended), wrestling (animals tumble and roll over each other as if to gain the dominant position), pinning (one animal stands over the other with its forepaws on the ventral surface of its opponent), and on-the-back (reciprocal behaviour of pinning, such that the animal is lying on its back with its ventral surface exposed) (Casto et al., 2003); *social investigation*, defined as body and anogenital sniffing; and *social rest*, measured when the subject is lying flat or standing still while maintaining physical contact with its partner, which may be either inactive or engaged in stationary behaviours (i.e. social sniffing, self- and social-grooming). Nonsocial behaviours included: *exploring*, defined as sniffing the air, rearing, and exploring cage; and *self-grooming*. Separate scores were obtained for each individual in test pairs but, since the two values are not independent of each other, pair means were used for statistical analyses. Thus, two pair means (one for each same-sex pair) were obtained per

litter. Following the social interaction test, animals were returned to standard paired housing conditions in the colony room where they remained undisturbed, except for regular animal care maintenance, until early adulthood.

Sucrose preference test. The SP protocol was adopted from(Kompagne et al., 2008). Beginning on PND70, rats underwent a five-day acclimatization period during which they were given two drinking bottles in their home cages: the first contained a 1% (weight/volume) sucrose/water solution, and the second was filled with tap water. The position of the bottles was switched daily to control for side preference. On PND75, the animals were transferred individually to fresh test cages and deprived of food and water for five hours. They were subsequently presented with a two-bottle choice sucrose preference test, with each bottle containing either tap water or the sucrose solution. Consumption of each solution was measured by weighing the bottles before and after the 60 min test. *Sucrose preference (SP)* was calculated according to the following equation *SP* = sucrose intake (g) / (sucrose intake (g) + water intake (g)) *100.

Adult open field test. On PND76, all rats underwent adult OFT. All animals were observed in the same open field apparatus as in the juvenile test, using the same testing procedures (with the exception that no heating blanket was placed under the adult cages during habituation). For each test, rats were again placed in the center of the novel arena, and allowed to explore the arena for 5 minutes. Before each test, and between tests, the arena was wiped clean using a 1% bleach cleaning solution and dried with paper towel. Each OFT-A session was video recorded as described above. Videos were again scored for emotionality (anxiety or fear) behavior, by scoring time spent in the center (s), as well as activity and exploratory tendencies (time spent grooming, frequency of line crosses,

and rearing frequency), using the same criteria defined in the juvenile OFT.

Adult forced swim test. On PND77, all rats underwent adult FST, according to a modified version of the original design proposed by Porsolt et al. (1978). The test was conducted in a 16" by 12" by 24" opaque plastic container such that the rats were not able to reach the floor or the rim of the container. Water temperature was maintained at 24±1°C, and the cylinder refilled with fresh water between tests. Adult FSPT was not performed as all rats were previously exposed to the FST as juveniles. The adult FST as again a 10 min test, and procedures for drying rats and recording sessions was identical to those described above. Rats were scored for the *duration of swimming (s)*, *climbing (s)*, *time spent immobile (s)*, and *latency to first immobility (s)*, as previously defined in the juvenile FST, as well as *diving time (s)*, defined as the time spent diving towards the bottom of the container and/or swimming underwater. All rats were returned to the colony room after adult FST and left alone for 24 hours before sacrifice.

2.2.6. Data manipulation and statistical analyses

All video-recorded tests were scored using Noldus Observer software (Noldus Observer software (version 10; Noldus Information Technology; Wegingen, The Netherlands), by a trained observer blinded to the experimental condition of each rat.

Statistical analyses were performed using SPSS 23.0 software. All results are shown as means ± standard error of the mean (S.E.M.) The effect of prenatal stress on each of litter size, average birth weight, and litter sex ratio was evaluated by means of separate one-way analyses of variance (ANOVA). Adult body weight (PND75) was analyzed using a two-way ANOVA, with prenatal treatment (NC, PPS) and postnatal condition (SC, EHC) as between subject factors. As fecal samples were collected during a

defined 120-min period, some dams (particularly in the NC condition) did not always produce samples in the allotted time. As a result, repeated measures ANOVA was not feasible for these data and univariate ANOVAs were used to analyze differences between Naïve and Prenatal Stress dams separately on each day at each time point.

Residual analysis was performed to test for the assumptions of all ANOVA analyses. Outliers were assessed by inspection of a boxplot, and extreme outliers (more than 3 Standard Deviations from the mean) were windsorized. Normality was assessed using Shapiro-Wilk's normality test for each cell of the design and homogeneity of variances was assessed by Levene's test. Any variables that failed tests of both normality and homogeneity of variance (p's < 0.05) were analysed using non-parametric tests. Due to the potential complicating factors of puberty, as well as a priori expectations of sex differences in behaviours (Young and Altemus, 2004; Altemus et al., 2014; Bangasser and Valentino, 2014; Korgan et al., 2014; Young and Pfaff, 2014) juvenile and adult males and females were analyzed using separate 2x2 ANOVAs with prenatal condition (NC, PPS) and postnatal condition (SC, EHC) as the between subject factors. Where significant overall effects were found in these ANOVAs, *post-hoc* simple main effects ANOVAs were performed for each behavioural measure to uncover the source of significance. Significant interaction effects were further analyzed using appropriate post-hocs. Details of other analyses are outlined in the appropriate section below. For all tests, the alpha level for the determination of statistical significance was set at $p = 0.05$.

2.3. Results

2.3.1. Dam fecal glucocorticoid levels

Analyses revealed that there were significant differences in fecal glucocorticoid levels between prenatally stressed dams and prenatally naïve control dams at all AM time points and 4/5 PM time points (See Figure 2.3). Dams subjected to PPS had significantly higher AM glucocorticoid levels than NC dams on GD 13, GD 15, GD18, GD20 (all p's ≤ 0.001). PPS dams also had higher PM glucocorticoid levels than control dams on GD15 and GD18 (p's ≤ 0.02), but there were no significant differences on GD13 ($p = 0.3$). Interestingly, on GD21, PM fecal glucocorticoid levels were significantly higher in control dams than in PPS dams ($p = 0.001$). When compared within-subjects on paired sample t-tests, in PPS animals, GD13 GCs were not significantly different from GD21 levels in either the AM ($p = 0.42$) or PM ($p = 0.34$) samples, suggesting no significant habituation to the stressor. Similarly, in NC animals, there were no significant differences between GD13 and GD20 in either AM or PM GCs.

2.3.2. Litter Characteristics at Birth

A multivariate ANOVA revealed that, on PND0, exposure to PPS resulted in lower average birth weight (g) in both male, $F(1, 17) = 5.38$, $p = 0.034$, $\eta_p^2 = 0.251$, and female $F(1,17) = 9.681$, $p = 0.007$, $\eta_p^2 = 0.377$ pups, compared to pups exposed to NC conditions prenatally (See Table 2.1). Neither litter sex ratio nor the size of the litter were significantly impacted by prenatal treatments (p's > 0.05; See Table 2.1).

2.3.2. PND15 Plasma Corticosterone

Prior to analyses, SPSS descriptive statistics were run to identify and exclude outliers greater than 2 SD from the mean, resulting in the exclusion of two samples from the NC/SC condition, and two samples from the PPS/SC condition, and one sample from the PPS/EE condition. Between subjects analyses revealed a significant impact of sex on plasma glucocorticoid levels $F(1,37) = 4.32$, $p = 0.045$, where females had higher corticosterone levels than males overall, as well as a trend for a Prenatal x Postnatal interaction $F(1,37) = 3.79$, $p = 0.059$. Given a priori expectations of sex differences (Pacak et al., 1998), we also analyzed data separately by sex. In males there was a significant effect of postnatal treatment ($p = 0.039$), where EHC increased corticosterone levels related to SC, this effect was driven by an increase in NC/EHC compared to all other groups (all p's ≤ 0.05). There were no significant effects of prenatal or postnatal treatment on corticosterone levels in females (see Figure 2.4).

2.3.3. Open field behaviour in juvenile (OFT-J) and adult (OFT-A) animals

i. Activity levels

Total Line Crosses

All data were first analyzed separately by age group and sex, in 2x2 ANOVAs with prenatal condition (NC vs PPS) and postnatal condition (SC vs EHC) as the between-subjects factors and total line crosses as the dependent variable. An analysis of the residuals was performed to test for the assumptions of the two-way ANOVA. One outlier in juvenile males and one outlier in adult females were windsorized. In juvenile males, there was a significant Prenatal x Postnatal interaction for line crosses $F(1,32) = 15.01$, $p < 0.001$, $\eta_p^2 = 0.319$. Simple main effects analyses revealed that for juvenile

males, PPS significantly decreased line crosses in PPS/SC compared to NC/SC ($p = 0.007$, $\eta_p^2 = 0.209$), while PPS significantly increased line crosses in PPS/EHC compared to NC/EHC ($p = 0.015$, $\eta_p^2 = 0.170$). Enhanced housing significantly decreased line crosses in NC/EHC compared to NC/SC ($p < 0.001$, $\eta_p^2 = 0.341$), but did not significantly change line crosses in PPS/SC compared to PPS/EHC. In juvenile females, there was a significant main effect of postnatal condition on line crosses $F(1,32) = 5.06$, $p = 0.03$, $\eta_p^2 = 0.137$, where EHC significantly decreased line crosses in juvenile females. In adult males, only a trend for a prenatal x postnatal interaction was present ($p = 0.063$, $\eta_p^2 = 0.128$; See Figure 2.5), where NC/EHC adult males had significantly less total line crosses than NC/SC adult males ($p = 0.035$).

Rearing Behaviour

An analysis of residuals was performed to test for the assumptions of the two-way ANOVA on time spent rearing (s). There was one outlier in juvenile females for juvenile rearing time that was windsorized. Juvenile females deviated significantly from a normal distribution for time spent rearing ($p < 0.05$). For each age group and sex, separate 2x2 ANOVAs with prenatal condition (NC vs PPS) and postnatal condition (SC vs EHC) as the between-subjects factors were performed. There were no significant effects of treatment on rearing behaviour in juvenile males or females, or in adult males using either ANOVA or a non-parametric Kruskal-wallis test (See Figure 2.6, all p's > 0.05). In adult females, there was a significant main effect of prenatal condition, where exposure to PPS decreased rearing time compared to NC animals, $F(1,30) = 5.15$, $p = 0.03$, $\eta_p^2 = 0.147$ (See Figure 2.6).

Grooming Behaviour

An analysis of residuals was performed to test for the assumptions of the two-way ANOVA on grooming time (s), with no significant outliers or violations of assumptions. ANOVA revealed no significant effects of treatment in juvenile or adult males or females (all p's > 0.05; See Figure 2.7).

Effects of Age on Activity Levels

A repeated measures MANOVA was performed analyzing the effects of age of testing (within-subjects) and the between-subjects factors of sex on the three dependent measures of activity/investigative tendencies (frequency of line crosses, time spent rearing, time spent grooming). Within-Subject analyses revealed a significant overall effect of Age on activity levels $F(3, 42) = 114.76$, $p < 0.001$, $\eta_p^2 = 0.891$ as well as significant Age x Sex interaction, $F(3,42) = 3.87$, $p = 0.016$, $\eta_p^2 = 0.217$. Breaking this down further by individual behaviours, we see that frequency of line crosses ($p < 0.001$, $\eta_p^2 = 0.874$ Figure 2.5) and time spent rearing ($p < 0.001$, $\eta_p^2 = 0.610$; Figure 2.6) increased significantly with age, where adults showed more episodes of behaviours relative to the juvenile period. An Age x Sex interaction was significant for time spent grooming, where adult females spent more time grooming than adult males ($p = 0.02$, $\eta_p^2 = 0.083$) with no differences in juvenile animals (Figure 2.7).

Effects of Dam GC levels on Activity Measures

Spearman's rho correlations between dam GC levels and offspring OFT activity behaviours were also examined (See Table 2.2). As with the ANOVA results, there was a significant negative correlation between dams' average AM GC levels and rearing time in adult females ($\rho = -0.367$, $p = 0.05$). Interestingly, there was also a significant negative

relationship between average AM GC levels and juvenile rearing in females ($\rho = -0.462$, $p = 0.015$), despite the lack of significant group differences in the ANOVA.

ii. Emotionality/anxiety behaviours

Center Time

An analysis of residuals was performed to test for the assumptions of the two-way ANOVA on OFT center time (s). There were two significant outliers in juvenile females, one significant outlier in juvenile males, and one significant outlier in adult females that were windsorized for analysis. Homogeneity of variance and normality assumptions were met for both juvenile and adult males and adult females (all p's > 0.05), though juvenile females were not normally distributed and did not demonstrate homogeneity of variable ($p = 0.03$). For each age group and sex, separate 2x2 ANOVAs were performed with prenatal condition (NC vs PPS) and postnatal condition (SC vs EHC) as the between-subjects factors and time spent in the center as the dependent variable. There were no significant effects of treatment condition on juvenile males, juvenile or adult females using either ANOVA or a non-parametric Kruskal-Wallis test (all p's > 0.05). In adult males, ANOVA revealed a significant main effect of prenatal condition, where exposure to PPS significantly decreased center time compared to NC animals, $F(1,27) = 5.6$, $p = 0.025$, $\eta_p^2 = 0.172$ (See Figure 2.8).

Freezing Time

An analysis of residuals was performed to test for the assumptions of the two-way ANOVA on OFT freezing time (s). There were two significant outliers in juvenile females, one significant outlier in juvenile males, and one significant outlier in adult females that were windsorized for analysis. Homogeneity of variance and normality

assumptions were not met for juvenile or adult males and females (all p's > 0.05) due to a large number of zero values in the data set. Episodes of freezing behaviour were therefore re-coded into categories, where freezing behaviour was classified as low/absent (0-19 seconds spent freezing) or moderate/high (20+ seconds spent freezing) and chi-square likelihood ratio tests were performed to examine the frequency of freezing behaviours following prenatal and/or postnatal treatment conditions. In juvenile females, exposure to prenatal stress significantly increased the number of offpring demonstrating 20+ seconds of freezing behaviour, $\chi2(1) = 6.05$, $p = 0.014$, without affecting freezing time in juvenile males. Interestingly, in adult females, postnatal exposure to EHC significantly decreased the number of offspring demonstrating freezing behaviour, $\chi2(1) = 4.179$, $p = 0.041$. Further analyses revealed that in adult females, PPS/SC had significantly more offspring exhibiting freezing behaviour than all other groups $\chi2(1) = 6.25$, $p = 0.01$, suggesting a 'rescue' by postnatal EHC in the PPS group is driving the overall effects of EHC in adult females (See Figure 2.9). Similarly, in adult males, the combination of PPS/SC also resulted in significantly increased frequencies of freezing behaviour compared to all other groups $\chi2(1) = 4.84$, $p = 0.03$ (See Figure 2.9).

Effects of Age on Emotionality

Repeated measures MANOVAs were performed to assess the effects of age of testing (within-subjects) and between-subjects factors of sex on a measure of emotionality/anxiety (time spent in the center, See Figure 2.8). Within-subject analyses revealed a significant overall effect of Age on center time $F(1, 44) = 19.67$, $p < 0.001$, $\eta_p^2 = 0.327$, where time in the center increased with age.

Effects of Dam GC levels on Emotionality Measures

Spearman's rho correlations revealed several relationships between dam GC levels and offspring anxiety behaviour (See Table 2.2). Consistent with the results found using ANOVA, there was a significant negative relationship between AVG AM GC and adult male center time in the SC condition ($\rho = -0.540$, $p = 0.046$), without significant effects in EHC adult males, or either EHC or SC adult females (See Table 2.2). As with the chi-square analyses, freezing time in juvenile females was significantly correlated with prenatal glucocorticoids, particularly on GD18 ($\rho = 0.4$, $p = 0.034$). In adults, again paralleling the chi-square results, both males ($\rho = 0.771$, $p = 0.001$) and females ($\rho = 0.746$, $p = 0.002$) exposed to the postnatal SC condition (but not EHC) showed a significant correlation between increased GC on GD18 and more freezing behaviour. In adult males, exposure to EHC actually resulted in an opposite relationship, where higher AVG AM ($\rho = -0.582$, $p = 0.018$) and PM ($\rho = -0.547$, $p = 0.026$) GCs negatively correlated with adult freezing behaviours (See Table 2.2).

2.3.4. Forced swim test behaviour in juveniles (FST-J) and adults (FST-A)

i. Immobility behaviours

An analysis of residuals was performed to test for the assumptions of the two-way ANOVAs on FST immobility, based on time spent immobile (s) and latency to first immobility (s). There was one significant outliers in juvenile females and one significant outlier in juvenile males on time spent immobile that were windsorized for analysis. For each age group and sex, separate 2x2 ANOVAs were performed with prenatal condition (NC vs PPS) and postnatal condition (SC vs EHC) as the between-subjects factors and time spent immobile (s) or latency to first immobility (s) as the dependent variables. When analyzed separately by age and sex, we see a significant main effect of EHC on

time spent immobile in juvenile females, where EHC decreased immobility overall $F(1,28) = 9.91$, $p= 0.004$. When adults were analyzed separately, no significant effects of condition were found (See Figure 2.10). In both juvenile males, $F (1,28) = 5.47$, $p = 0.027$, $\eta_p^2 = 0.163$, and juvenile females $F (1,28) = 6.25$, $p = 0.019$, $\eta_p^2 = 0.200$, there was a significant main effect of prenatal treatment, where PPS signficiantly decreased the latency to first immobility $F(1,25) = 0.019$. In juvenile females, postnatal EHC also had a significant main effect, increasing latency to first immobility $F(1,28) = 6.92$, $p = 0.014$, $\eta_p^2 = 0.217$. There were no significant effects of treatment condition on latency to first immobility in the adult FST (See Figure 2.11). Repeated measures ANOVAs were also performed to analyze the effects of age of testing (within-subjects) on measures of 'depressive' behaviours (time spent immobile and latency to first immobility) in the FST (See Figure 2.10 and Figure 2.11). Within-subject analyses revealed no significant impacts of age ($p > 0.05$).

Interestingly, spearman's rho correlations demonstrated several significant correlations between dam GC levels and behaviour (See Table 2.2). Despite the lack of group differences, in juvenile females there was a significant positive correlation between more time spent immobile (s) and AVG AM GC ($\rho = 0.452$, $p = 0.02$), and AVG PM GCs ($\rho = 0.41$, $p = 0.036$). In adult females, there were significant correlations in the opposite direction, despite the lack of group effects, where increased AVG AM GCs were correlated with an increased latency to first immobility ($\rho = 0.377$, $p = 0.044$). When analyzed separately by postnatal condition, we see several interesting correlations in males exposed to EHC, where AVG AM GC were significantly negatively correlated with juvenile FST immobility ($\rho = -0.726$, $p = 0.003$), and followed a similar trend in

adult animals ($\rho = -0.428$, $p = 0.056$). AVG PM GC levels were also significantly negatively correlated with latency to first immobility in juvenile ($\rho = -0.523$, $p = 0.05$) and adult ($\rho = -0.601$, $p = 0.014$) EHC males. See Table 2.2.

ii. Activity in the forced swim test

An analysis of residuals was performed to test for the assumptions of the two-way ANOVAs on FST activity, based on swimming (s) and climbing (s). There was one significant outlier for juvenile females in climbing behaviour, and two significant outliers in adult females in climbing behaviour that were windsorized for analysis. For each age group and sex, separate 2x2 ANOVAs were performed with prenatal condition (NC vs PPS) and postnatal condition (SC vs EHC) as the between-subjects factors and time spent climbing (s) or time spent swimming (s) as the dependent variables. When analyzed separately by age and sex, there were no significant effects of prenatal or postnatal conditions on juvenile swimming or climbing behaviours, or behaviours in adult females (all p's > 0.05). There was a significant main effect of prenatal stress on adult male climbing behaviours $F(1,34) = 4.21$, $p = 0.048$, $\eta_p^2 = 0.110$ (See Figure 2.12) . Repeated measures MANOVAs were performed to analyze the effects of age of testing (within-subjects) on measures of FST active behaviours (swimming and climbing). The only significant effects revealed were a main effect of Age on swimming $F(1,54) = 8.57$, $p = 0.04$, , $\eta_p^2 = 0.137$, where swimming decreased with age (See Figure 2.11).

Spearman's rho correlations revealed significant correlations between dam GC levels and adult FST activity (see Table 2.2). There was a significant correlation between higher average AM GC levels and more adult female climbing behaviour ($\rho = 0.444$, $p = 0.017$), which when analyzed by postnatal condition, was significant in adult EHC

females ($\rho = 0.491$, $p = 0.05$), but not SC. In males, increased GCs on GD 18 AM was associated with decreased climbing behaviour ($\rho = -0.557$, $p = 0.001$), and remained significant in both SC ($\rho = -0.635$, $p = 0.015$), and EHC conditions ($\rho = -0.491$, $p = 0.05$). See Table 2.2.

2.3.5. Peri-adolescent social behaviour

An analysis of residuals was performed to test for the assumptions of the two-way ANOVAs on social interaction behaviours, based on time spent in: social sniffing, rough and tumble play, social rest, cage exploration and grooming (s) during the social interaction test. In juvenile females, there were three significant outliers for rough and tumble play behaviour, and two significant outliers in social sniffing and grooming behaviour and one male outlier in each of grooming and sniffing behaviour that were windsorized for analysis. A violation for homogeneity of variance was found for females in for rough and tumble play behaviour ($p = 0.001$), likely due to a large number of zero values. ANOVAs were used to examine the between-subject factors of Prenatal Condition, Postnatal condition, on a series of social behaviours present during a social interaction test, including social sniffing, rough and tumble play, and social rest, as well as the anti-social behaviours of cage exploration and self-grooming in males and females.

Social Sniffing

There were no significant effects of prenatal or postnatal conditions on social sniffing behaviour (all p's > 0.05).

Rough and Tumble Play

ANOVA revealed that there was a significant main effect of prenatal condition on male rough and tumble play behaviour $F(1,37) = 7.54$, $p = 0.01$, $\eta_p^2 = 0.182$, where PPS

significantly decreased rough and tumble play. There were no significant effects in females. Notably, there was also a significant main effect of sex, $F(1,62) = 13.1$, $p = 0.001$, where males had higher levels of rough and tumble play than females overall (See Figure 2.12).

Social Rest

Significant interactions between prenatal and postnatal condition were found in both males, $F(1,37) = 12.466$, $p = 0.001$, $\eta_p^2 = 0.268$, and females, $F(1, 33) = 4.41$, $p = 0.04$, $\eta_p^2 = 0.128$, though the effect size was larger in males. No significant post-hoc effects could be resolved in females. Using the Tukey's HSD correction factor, post-hoc comparisons revealed that PPS/SC males had significantly higher levels of social rest than PPS/EHC males ($p = 0.009$), as well as a trend towards an increase relative to NC/SC ($p = 0.07$). PPS/EHC males also showed a trend towards an decrease relative to NC/EHC males ($p = 0.067$) in social rest (See Figure 2.13).

Social Exploration

Significant interactions between prenatal and postnatal condition were found in both males, $F(1, 37) = 24.23$, $p < 0.001$, $\eta_p^2 = 0.416$, and females, $F(1,33) = 9.46$, $p = 0.004$, $\eta_p^2 = 0.24$, , though the effect size was larger in males. In males, post-hoc analyses with a Tukey's HSD correction factor revealed that PPS/EHC males spent significantly more time in exploration than PPS/SC ($p = 0.001$) or NC/EHC males ($p = 0.001$). A similar pattern was found in females, where PPS/EHC females spent significantly more time in social rest than NC/EHC females ($p = 0.022$; See Figure 2.13)..

Self-Grooming

In females, but not males, there was a significant main effect of prenatal condition on grooming behaviour $F(133) = 4.48$, $p = 0.043$, $\eta_p^2 = 0.13$, wher PPS significantly increased grooming behaviour (See Figure 2.13).

Correlations between Prenatal GC and Social Behaviours

Spearman's rho correlations revealed several correlations between dam GC levels and social behaviours (see Table 2.2). Consistent with ANOVA, in males, increased AVG PM GCs was significantly correlated with decreased rough and tumble play ($\rho = -0.379$, $p = 0.039$). Despite the lack of overall group effects, there was a significant correlation between higher AVG PM GCs and females exposed to EHC ($\rho = -0.669$, $p = 0.009$). Overall, in males, higher average PM GC levels were related to less exploration ($\rho = -0.410$, $p = 0.024$), and more social rest behaviour ($\rho = 0.426$, $p = 0.019$). When we analyzed SC and EHC animals separately, we observed that average AM and PM GC levels correlated with exploration in opposite directions, where in SC animals, higher AM and PM correlated with less exploration ($\rho = -0.691$, $p = 0.006$ and $\rho = -0.602$, $p = 0.023$, respectively), while in EHC, higher AM GC levels correlated with more exploration ($\rho = 0.609$, $p = 0.012$). See Table 2.2.

2.3.6. Sucrose preference test

An analysis of residuals was performed to test for the assumptions of the two-way ANOVAs on sucrose preference. There was one significant outlier in females, and one outlier in males, for sucrose preference percentage that were windsorized for analysis. Homogeneity of variance and normality assumptions were violated for both males and females (all p's < 0.05). Therefore, a non-parametric Kruskal-wallis test was used for both males and females across 4 conditions (NC/SC, NC/EHC, PPS/SC, PPS/EHC). In

females, there was a significant effect of treatment condition $H(3) = 13.55$, $p = 0.004$. Posthoc analyses revealed PPS/SC females had a significantly lower preference for sucrose relative to all other female groups (PPS/EHC, NC/SC, and NC/EHC, all p's ≤ 0.05), while there were no significant group differences in male sucrose preference (See Figure 2.14).

Consistent with group differences, Spearman's rho analyses revealed a significant negative correlation between average AM GCs and sucrose preference in females ($\rho = -0.349$, $p = 0.05$), but not in males, when the sexes were analyzed separately. Further, this negative correlation between sucrose preference and average AM GCs was present in SC females ($\rho = -0.703$, $p = 0.005$), but not in EE animals (See Table 2.2).

2.3.7. Relationships between juvenile and adult behaviour

i. Juvenile open field activity predicting adult behaviours

Spearman's rho correlations were used to examine relationships between juvenile OFT measures of activity/investigative tendencies (line crosses, rearing, grooming) on all adolescent and adult behaviours to determine if juvenile OFT behaviours were predictive of adolescent and adult behavioural responses. Overall, juvenile line crosses were not significantly correlated with any adult behaviours in either males or females. In juvenile females, increased juvenile rearing behavior was correlated with more immobility on the adult FST ($\rho = 0.386$, $p = 0.027$), and less adult FST climbing ($\rho = -0.395$, $p = 0.023$). In males, juvenile rearing behaviour was also correlated with adult FST activity, where more rearing was correlated with a shorter latency to first immobility ($\rho = -0.413$, $p = 0.017$), and less swimming behaviour ($\rho = -0.389$, $p = 0.023$). In males, juvenile OFT grooming behaviour was associated with an increased adult FST latency ($\rho = 0.461$, p =

0.007), and increased sucrose preference percentage (ρ = 0.393, p = 0.022). See Table 2.3.

ii. Juvenile open field anxiety predicting adult behaviours

Spearman's rho correlations revealed that, in females, juvenile center time in the OFT correlated with less adult FST climbing (ρ = -0.376, p = 0.03). Juvenile freezing in females on the OFT was correlated with less rearing in the adult OFT (ρ = -0.466, p = 0.007), along with less adult FST immobility (ρ = -0.470, p = 0.006), a longer latency to first immobility (ρ = 0.457, p = 0.008), and more time spent swimming (ρ = 0.405, p = 0.019). In males, juvenile freezing behaviour in the OFT was correlated with less adult FST immobility (ρ = -0.359, p = 0.037). See Table 2.3.

iii. Juvenile forced swim behaviours predicting adult behaviours

Spearman's rho correlations revealed that overall higher juvenile FST immobility in females correlated with decreased adult OFT rearing (ρ = -0.362, p = 0.05), and decreased exploration (ρ = -0.556, p = 0.003) in the adolescent social interaction test. Interestingly, despite the lack of correlation with adult FST immobility, in females, a longer latency to immobility in the juvenile FST correlated with a significantly increased adult sucrose preference (ρ = 0.376, p = 0.04).. In males, more juvenile FST immobility (ρ = 0.387, p = 0.042), and a shorter latency to first immobility (ρ = -0.43, p = 0.023) correlated with more freezing time on the adult OFT. See Table 2.3.

iii. Peri-adolescent social behaviours predicting adult behaviours

Spearman's rho correlations revealed that in males, overall higher levels of juvenile play behaviour (ρ = -0.331, p = 0.048) and lower levels of exploration (ρ = 0.378, p = 0.023) were correlated with decreased FST swimming. In males, more social

play behaviour was also significantly correlated with lower sucrose preference (ρ = -0.346, p = 0.039). In females, higher levels of exploration in the social behaviour test correlated with more adult rearing in the OFT (ρ = 0.388, p = 0.041). See Table 2.3.

2.4. Discussion

Overall, we see that our novel model of prenatal predator stress significantly increased circulating glucocorticoids in the mother throughout pregnancy. However, this prenatal predator stress (PPS), and subsequent postnatal enhanced housing condition (EHC), had varied, often sex- and age-specific effects on behavior. Many of the effects of group effects of PPS emerged in adolescence/adulthood. In adult males, PPS significantly decreased center time in the open field test (suggesting increased anxiety behaviour) and this was not affected by postnatal EHC. Similarly, PPS increased OFT freezing (also suggesting increased anxiety). This was prevented by postnatal EHC, and was found in both males and females. In adult females, PPS significantly decreased sucrose preference (suggesting anhedonia) and this effect was mitigated by postnatal EHC. PPS also PPS also showed a strong suppressive effect on adolescent male rough and tumble play in the social interaction (SI) test. In some cases, despite a lack of significant group effects of prenatal stress, prenatal fecal GC were still significantly correlated with behaviours, suggesting an important role for the individual dam's response in later anxiety/depressive behaviours. Unlike PPS, where most effects emerged later on (with the exception of latency to first immobility in the juvenile FST, and line crosses in the OFT for males), some of the effects of EHC in juvenile females (decreasing FST immobility and decreasing line crosses in the OFT) were no longer present in adults. When the effects of PPS and EHC interacted, the majority of the effects (6/7 behaviours) were preventative –

that is, the combination of PPS/EHC prevented effects of either PPS or EHC alone (OFT juvenile and adult male line crosses, OFT-A male and female freezing, SI M social rest, female sucrose preference). In the remaining case (1/7; SI exploration), EHC and PPS exerted similar, independent effects on behaviour, but the combination resulted in significant effects in the opposite direction (See Table 2.4 for a summary of group effects). Juvenile behaviours were not significantly correlated with the same adult behaviours (i.e. - juvenile rearing predicting adult rearing, etc), although juvenile OFT rearing and freezing, along with juvenile FST immobility showed promise at predicting other anxious or depressive behaviours in adulthood. These effects will be discussed in detail below.

2.5.1. Physiological Measures

Prenatal Predator Exposure and Dam GC Levels

Our novel, ethologically relevant model of PPS increased AM GC levels on each day of exposure, as well as PM GC levels on GD15 and 18 compared to animals that did not experience PPS (though GC levels were higher in NC than PPS on GD21). Overall, this suggests our PPS exposure is significantly increasing circulating GCs in pregnant dams compared to controls, and that this HPA response is still present at the end of pregnancy, where both AM and PM GC levels were similar to those measured on GD13 in prenatally stressed animals. It is initially surprising that AM GC levels were elevated on the first day of the stressor (GD13), as literature on the basal metabolic pattern of glucocorticoids suggests that levels in the feces should correlate most strongly with levels in the blood from approximately 6-9 hours earlier (Touma et al., 2004; Cavigelli, 2005). However, excretion of injected corticosterone metabolites suggest that time of day is

important for the rate of excretion, with peak metabolites present at 4-6 hours (and a significant portion already present within 2 hours) in the dark phase of the rat's circadian cycle (Touma et al., 2003). As we allowed up to 2 hours following the end of the stressor to collect fecal samples, and collected samples during the dark cycle, this shorter time window could explain our increase in AM GC levels on the first day. The significant increase in GC levels in NC dams relative to PPS on GD21 is also initially surprising, but likely caused by a normal pre-birth surge in corticosterone (Brunton and Russell, 2008). Though we would expect to see this in PPS dams as well, PPS rats had a (non-significant) increase in average gestational length, which suggests this slightly later surge may not have been captured by fecal sampling on GD21 for this group. Alternatively, as predator stress is known to alter aspects of the stress response in non-pregnant animals, it is possible that our repeated stress is preventing or blunting the normal rise in corticosterone prior to birth. Though an analysis of the full diurnal rhythm was beyond the scope of this study, in NC animals, fecal GC levels increased over the day, with AM levels lower than PM levels, and this was likely due to the increase in plasma corticosterone levels at the beginning of the dark cycle, 8-10 hours earlier. In PPS animals, levels were higher in the AM than PM, potentially reflecting changes to basal corticosterone rhythms. Regardless, it is clear that prenatal predator stress is generating a significant physiological response in dams throughout the exposure period.

PND15 Plasma GC Levels

A subset of pups that were not exposed to any behavioural testing were sacrificed on the same day as the start of behavioural testing (PND15), and circulating levels of GC were analyzed. Overall, female pups had significantly higher corticosterone levels than

male pups on PND15, regardless of condition, as has been previously reported (Patchev et al., 1999). Prenatal stress did not elevate basal levels of corticosterone in pups in either sex, which has also been found in previous literature (Henry et al., 1994). Interestingly, in male pups, but not females, EHC increased basal corticosterone levels relative to other groups. Though environmental enrichment is often thought of as a way to prevent/repair negative stress-related effects, studies have shown that adolescent enrichment increases basal circulating corticosterone levels in male guinea pigs, acting independently from prenatal chronic mild stress (Emack and Matthews, 2011). Environmental enrichment also increases baseline anxiety behavior in some studies, while acting as a mild 'inoculation' stressor against other chronic stressors (Connors et al., 2015). Similarly, a 6-week adult enrichment paradigm has been shown to increase basal corticosterone levels in Long-Evans rats, but it also facilitated a faster return to baseline after stress (Konkle et al., 2010), supporting the idea of enrichment as a mild stress that buffers against other larger stressors. As the increase in GC was not present in the PND15 PPS/EHC males, it is possible that our prenatal predator stressor interfered with the effects of EHC on circulating corticosterone levels, without causing any obvious independent effects on PND15 corticosterone levels.

2.5.2. Open Field Test

In adults, PPS significantly decreased center time in male rats and EHC did not rescue these effects. This decreased center time in males suggests a more anxious phenotype, and is consistent with the results of several studies, where prenatal stress significantly increased anxious behaviours in males, but not females (Barna et al., 2003; Zuena et al., 2008; Brunton, 2013), though notably this effect is not always reported

(Zagron and Weinstock, 2006; Aziz et al., 2012). In the present study, this increase in anxious behavior was not found in juvenile males, suggesting that either center time is not useful in assessing juvenile anxiety, or that this anxiety only emerged following puberty. Supporting the idea of anxious behaviours emerging after puberty in males, adult (but not juvenile) males, in the PPS/SC condition also showed significant increases in time spent freezing compared to other conditions, another indicator of 'anxious' behavior, as a danger avoidance strategy. Interestingly, however, unlike center time, this behavior was prevented/restored by EHC. In females, despite the lack of effects on OFT center time, both juvenile and adult females demonstrated increased freezing time, suggesting that freezing behaviour and avoiding the center of the arena may be different strategies, both representing a response to anxiety, that could be differentially employed in males and females. Consistent with an effect of fetal exposure to GC on 'anxiety' behaviour, adult freezing time was significantly (positively) correlated with GD18 AM GC levels collected from dams during pregnancy for both males and females, and with center time in adult males. Previous research suggested that prenatal dexamethasone treatment could induce OFT anxiety in adult animals (postnatal week 10), but not in animals during the peri-pubertal period (postnatal week 4) (Nagano et al., 2008), and our results suggest this is also true for prenatal predator stress effects on males. Adult females demonstrated no significant changes in their OFT center time, suggesting that they were less sensitive to some of the effects of PPS on anxious behaviors, at least as assessed by this task. However, despite the lack of overall group differences following PPS, juvenile females did show a decrease in center time with higher GD18 AM GCs, along with the correlation between higher GD18 GCs and more freezing behaviour that paralleled ANOVA results.

This suggests a heightened stress response of dams on the 6th day of stress exposure (perhaps suggesting less habituation in these dams) was associated with an increase in female's likelihood of anxious behaviours, particularly in the juvenile period.

These sex and treatment differences in OFT-A anxiety behavior are not likely due to differences in overall activity levels, as PPS did not significantly alter adult line crosses, rearing, or grooming behavior. Interestingly, however, despite a lack of overall group effects of PPS, a significant correlation was found between higher GD18 AM GC levels and less adult rearing, particularly in SC conditions. One recent study of repeated variable prenatal stress reported a significant decrease in adult OFT rearing behaviour in both sexes (Wilson et al., 2013), which they suggest to be related to less 'goal-directed' exploratory behaviour, or increased anxious/neophobic behaviours, rather than locomotor activity. However, other studies have suggested higher rearing activity is related to greater locomotor activity (Borta and Schwarting, 2005) and indicative of a stronger reaction to novelty (Thiel et al., 1999; Pawlak and Schwarting, 2002). Importantly, relationships between rearing and exploration/locomotion can depend on the type of stressor employed (Pijlman et al., 2003), as well as post-weaning environment (Brenes et al., 2009), which suggests that different goals/underlying mechanisms can trigger a suite of behaviours, some of which overlap across strategies. That is, rearing, or line crosses, or grooming, depending on the context – could indeed be either indicative of exploration, anxiety, or hyper-responsivity to novelty, and should be considered in the context of other behaviours exhibited by the animal, as well as the type of stressors employed.

PPS significantly decreased line crosses in juveniles, with no significant effects in adult male rats, which is in contrast with some recent work suggesting increased adult

(Wilson et al., 2013) and juvenile (Gué et al., 2004) activity following prenatal stress, though these differences could be attributed to different stressors schedules, strains, or time of testing. Interestingly, line crosses were significantly decreased by EHC, but the combination of PPS and EHC interfered with (prevented) the effects of either alone, returning levels to those similar to NC/SC.

While activity (locomotion) in the open field is not necessarily related to anxiety behaviours, as it is not consistently affected by benzodiazepines (Choleris, 2001), some studies suggest it is an important measure of habituation (Yen et al., 2013). Indeed, raising rats in isolation or impoverished conditions (Heidbreder et al., 2000), or neonatal immune activation (Rico et al., 2010) may result in 'hyperactive' locomotion, and this hyperactivity can be prevented or reduced by physical or social enrichment (Neugebauer et al., 2004; Hannan and Kalueff, 2010; Horvath et al., 2013; Mosaferi et al., 2015). This is consistent with our decrease in juvenile and adult line crosses following EHC, though we did not find a corresponding decrease in adult rearing in males, as reported by others (Kalueff and Tuohimaa, 2004; Mosaferi et al., 2015). These differences in effects on rearing could be attributed to differences in the timing of enrichment, as both Kaleuff et al. (2004), and Mosaferi et al. (2015), used post-weaning enrichment.

It is interesting to note that in our study, the combination of PPS and EHC prevented some of the effects of either PPS or EHC alone. The combination of PPS and EHC prevented effects on juvenile line crosses and adult freezing time. Some recent research suggests that EE during the prenatal period may actually be a mild stressor, increasing freezing behavior in the elevated plus maze, and decreasing sucrose preference – though it also appears to provide a resiliency to chronic stress, where these same effects

actually recovered in the presence of repeated adult stressors (Cymerblit-Sabba et al., 2013).

Importantly, many of the effects of EHC in the OFT were specific to the juvenile period, with only adult freezing showing a significant interaction between EHC and PPS. Further, neither juvenile line crosses nor juvenile grooming behaviour significantly predicted adult behaviours, suggesting reported changes in juvenile line crosses and rearing may reflect aspects of a juvenile specific strategy to the novel environment, and be less useful as consistent trait-specific indices of response to novelty or anxiety across both juvenile and adult periods. These juvenile specific effects of EHC are consistent with a recent study suggesting the prenatal EE may have greater effects in young males than adults, and that these effects may be dependent on the strain (and stress reactivity) of the dam (Rosenfeld and Weller, 2012).

Juvenile rearing behaviour, unlike grooming or line crosses, was a significant predictor of adult behaviours, including greater immobility and less swimming or climbing behaviour in the FST-A, suggesting that juvenile rearing may be an early life indicator of both higher activity levels and increased emotionality in adults in this model. Similarly, juvenile center time correlated positively with less climbing behaviour in the adult FST, which is often considered a marker of 'active coping' (discussed in the next section in more detail). This increase in juvenile 'anxiety' predicting adult active coping is particularly intriguing when we consider that in many cases, anxiety and depression are co-morbid disorders, and that in some clinical studies, symptoms of early life anxiety are associated with both later anxiety disorders and depression in adolescence (Andersen and Teicher, 2009). Interestingly, although juvenile rearing was not significantly altered by

PPS in our study, there were significant correlations between the dam's GD18 GC response in the morning (AM) and both juvenile and adult rearing behaviour, suggesting individual differences in the dam's corticosterone response or subsequent behavior could also be contributing the role of this behaviour in predicting adult outcomes. Together, these results suggest that rearing in the juvenile OFT could be an important marker predicting later adult anxiety/depression in rodent models, and that, despite a lack of overall group differences, some underlying neurophysiological changes may already be present at our young time point (PND15).

2.5.3. Forced Swim Test

In adults, there were no significant impacts of condition on immobility behaviour, unlike previous studies demonstrating that prenatal restraint stress (Abe et al., 2007) or dexamethasone treatment (Oliveira et al., 2006) increased immobility, though this result is not always found (Nagano et al., 2008). PPS did significantly decrease climbing behaviour in adult males on the FST, however, suggesting that the prenatal stress exposure may alter coping strategies in the FST, without necessarily triggering behavioural 'despair' (Detke et al., 1995; Barros and Ferigolo, 1998). Interestingly, despite the lack of overall group effects of PPS, there was a negative correlation between adult immobility and GD18 AM prenatal GC levels, suggesting that, with less habitutation, prenatal GC may have actually decreased adult immobility. Interestingly, GD15 PM GC levels were significantly correlated with increased immobility in EHC animals, suggesting a stronger early response to the repeated stressor may have contributed to the later behavioural despair in offspring of individual dams. Recent research has suggested the effects of prenatal EE may have opposite effects on adult FST

immobility depending on the strain of rat used, where immobility was increased in Wistar rats, and decreased in Wistar Kyoto rats (a genetic model of depression; (Rosenfeld and Weller, 2012). The authors suggest that this could be due to differences in the way the dams respond to the EE condition, or alternatively that underlying genetic differences may be stable and difficult to change with (fairly brief) early life interventions. It may also be the case in our model, where perhaps dams with the strongest stress reactivity to prenatal predator exposure, had a stronger (more aversive) response to the postnatal EHC condition, creating a more negative environment. Alternatively, dams with the highest prenatal GCs may have caused the largest changes to the offspring, and these changes could not be mitigated with our postnatal EHC treatment.

Unlike adulthood, in juveniles we again saw significant effects of both PPS and EHC, where EHC significantly decreased immobility in females, regardless of prenatal condition. This is consistent with a recent study suggesting that the effects of EHC may be more prominent in females and younger animals (Rosenfeld and Weller, 2012). This again points to the possibility that our postnatal EHC was insufficient to prevent/rescue many of the effects of PPS over the long term, and perhaps this type of postnatal intervention may have a lesser capacity to shape the brain than prenatal PPS. Similarly, recent research has found that a maternal separation stressor induced learned helplessness in juvenile females, but this effect was insufficient to persist into adulthood (Leussis et al., 2012), and adolescent EE has been unable to prevent/reverse a number of changes in the brain induced by prenatal nicotine exposure (Darbra and Pallarès, 2011), although it had a number of independent effects. In juvenile females, PPS also significantly decreased the latency to first immobility, suggesting this may be a more sensitive

measure of 'despair' than total immobility in juvenile females. Despite the lack of overall group effects, AVG AM GCs were associated with more immobility in juvenile females, and a shorter latency tomore immobility in the juvenile FST, again supporting a role for PPS in juvenile FST immobility in females, though effects were either absent or in the opposite direction in adulthood. Interestingly, increased immobility in the juvenile FST correlated with opposite effects on adult OFT freezing behaviour (less freezing in adult females, more in adult males), suggesting that in juveniles, the FST may be an important sex-specific predictor of adult anxiety behaviours (rather than directly impacting 'depression'), and that some of the underlying neurophysiological changes in anxiety or stress responding may already be present at this young age.

2.5.4. Social Interaction test

Social behavior is considered a rewarding experience for rats (Humphreys and Einon, 1981), and reductions in social play are related to juvenile depression in some models (Malkesman and Weller, 2009). Consistent with previous research using prenatal restraint stress (Morley Fletcher et al., 2003), or prenatal LPS (Taylor et al., 2012), our model of prenatal predator exposure significantly decreased rough and tumble play behavior in peri-adolescent male rats, but not females. Unlike previous research using adolescent enrichment (Morley Fletcher et al., 2003), postnatal EHC did not rescue these effects on rough and tumble play, nor did it appear to impact rough and tumble play independently. Thus, as with FST and OFT behaviours, our postnatal EHC intervention was insufficient to prevent/rescue the effects of PPS on play behaviour in males, in contrast to later EE interventions, such as the post-weaning EE used by Morley-Fletcher and colleagues (2003), which has such effects.

Previous research suggests that rats bred for high anxiety tend to spend less time in active, but not passive, social interactions and these different types of play behaviours are likely associated with different neural mechanisms (Pawlak et al., 2008). Indeed, rough and tumble play is highly sexually dimorphic, and levels of play in male offspring can be decreased by prenatal exposure to the anti-androgen flutamide (Casto et al., 2003), as well as by prenatal stress (Ward and Weisz, 1984); thus, disruptions of these behaviours may be more of a marker of prenatal stress experience in males than a marker of decreased reward (anhedonia) to social play. Rough and tumble play is sometimes interpreted as aggressive behaviour (Parent and Meaney, 2008), and may not always reflect better social interactions, as recent research suggests that peri-pubertal stress actually increased adolescent play fighting, but decreased an adult preference for social exploration (Veenit et al., 2013), and both postnatal handling (Edelmann et al., 2013) and high postnatal licking and grooming by dams (Parent and Meaney, 2008), believed to be related to 'better' adult stress responding, decreased juvenile rough and tumble play behaviour.

Similar to another study of variable prenatal stress, we also found that PPS increased social resting behaviour in males (Schroeder et al., 2012). The patterns of these results is consistent with work by Morley-Fletcher and colleagues that showed post-weaning EE reduced exploration and increased social rest, while the combination of PS/EE interfered with this increase (Morley Fletcher et al., 2003). These results are interesting in that they suggest our postnatal EHC may have had similar patterns of effects on social interaction as those seen by Morley Fletcher et al. (2003). Differences in effects of PPS could be attributed in part to the different types of stressors used (restraint

vs. predator), or the rodent strain used (Sprague Dawleys vs Long Evans), as well as the timing of the EHC. Indeed, Schroeder and colleagues (2012) found significant increases in social behaviour following repeated variable stress, but not repeated restraint, in Wistar rats - suggesting that PPS, like variable repeated stress, may produce different (more ethologically relevant?) changes to adolescent social behaviour. Interpretations of these behaviours in light of anxiety/depression are more complex, as we did not find many correlations between the social interaction test and adult behaviours, with the exception of increased rough and tumble play behaviour relating significantly to decreased FST swimming and decreased sucrose preference in adult males. These results are particularly interesting as research on postnatal handling suggests that the decrease in juvenile play behaviour following neonatal handling was associated with increases in serotonin signaling (Edelmann et al., 2013), which has also been associated with FST swimming behaviour and depression (Cryan et al., 2005).

2.5.5. Sucrose Preference Test

In our two-bottle choice task of sucrose preference, PPS significantly decreased sucrose preference in adult females, but not males, and EHC returned these effects to normal levels, while EHC alone appeared to have no significant effects on preference. This is consistent with some recent research suggesting sucrose preference is not altered by postnatal EE or communal nesting alone (Connors et al., 2015), or by postnatal maternal separation (Feng et al., 2014) but as in our case, EHC only acts to reverse effects of PPS. Recent research has also suggested that while prenatal EE alone increased offspring anhedonia, it acted to prevent anhedonia following adult stress exposure (Cymerblit-Sabba et al., 2013), as did a communal nesting environment (Branchi et al.,

2011). Sucrose preference is reported to decrease following chronic stress or corticosterone injection and is often interpreted as a decrease in the 'pleasure' associated with consuming a sweet solution (Jayatissa et al., 2006), though effects of antidepressants on sucrose anhedonia are less consistent in females (Green et al., 2009; Carrier and Kabbaj, 2012b).

The effects of prenatal stress on anhedonia have been less consistent, though some recent research shows that prenatal restraint stress in mice can decrease sucrose preference in females, but not males (Behan et al., 2011). This effect was only triggered following a 'second hit' of adult chronic mild stress in rats (Van den Hove et al., 2014), while conversely, prenatal immune activation decreased sucrose preference in both male and female mice (Bitanihirwe et al., 2010). Further, stress during early gestation has been reported to have significant effects on male sucrose preference (Mueller and Bale, 2008), with no differences in depressive symptoms between the sexes following late gestational stressors. Clearly, both timing and type of stressor, as well as strain and species, are likely contributing to these contradictory findings of the interactive effects of prenatal stress and sex on anhedonia.

Anhedonia is considered one of the most important endophenotypes of depression, with high levels of heritability and familial association. Anhedonia may also be a stable trait marker for vulnerability to depression, even when individuals are not in an active episode, though the trait is also found in some other neuropsychiatric disorders, including schizophrenia (Pizzagalli, 2014). Given our finding of decreased sucrose preference, but not increased FST immobility in adulthood, it may be that our PPS female offspring are demonstrating an underlying marker for later vulnerability to depressive

disorders, perhaps following a 'second hit' of adult stress (Van den Hove et al., 2014), rather than demonstrating a current 'depressed' phenotype. Alternatively, our model of prenatal stress may contribute only to some of the behaviours/symptoms under the umbrella of clinical depressive behaviours. Interestingly, higher rates of anhedonic symptoms have been reported among depressed adolescents who experienced severe childhood adversity, (Lumley and Harkness, 2007), which suggests that early life stressors could increase this symptom in particular, as we are also seeing in our model. Further, both increased juvenile FST immobility, and a trend towards decreased open field center time in juveniles were correlated with lower sucrose preference, suggesting that juvenile responses on the FST or OFT could be important markers of later depressive vulnerability, even when no group differences are visible in juvenile behaviour.

2.5.6. Conclusions

Together, these results reveal a significant impact of PPS on the developmental trajectory of anxious and depressive behaviours. Our model incorporating PPS and EHC produced distinct sex and age specific effects, with adult males showing an anxious phenotype, with significant OFT anxiety, while females demonstrated an adult anhedonia with some changes to adult anxiety and juvenile FST immobility. Many of the effects of PPS were not visible in the juvenile behavioural tasks, emerging as significant group differences only in adulthood, which mirrors the human literature where reported rates of anxiety and depressive disorders increase dramatically during adolescence and early adulthood (Kessler et al., 2001). Further, using this model, we demonstrated the importance of individual variability in response to stressors, as dam fecal GC levels significantly correlated with behavioural outcomes, even in cases where group

differences were unable to be resolved. Thus, our PPS model may be a particularly useful ethological model of prenatal stress, as not all individuals exposed to early life stress go on to develop disease. In particular, the individual responses of dams on GD15, the 3rd day of stress exposure, and GD18, the 6th day of stress exposure, appeared to play an important role in adult behavioural responses, though further research is required to tease apart the importance of underlying genetic variability to stress and the impact of these stressors on later dam maternal behaviour (Smith et al., 2004; Stolzenberg and Champagne, 2015). Surprisingly, exposure to PPS in our model did not result in many significant effects on juvenile behaviour, with some social behavioural changes emerging in adolescence, and most appearing in adulthood. Despite this, some juvenile behaviours were strong predictors of later adult anxiety and depression, with juvenile OFT rearing emerging as a predictor of more adult freezing and rearing, as well as greater FST immobility, while juvenile FST immobility related to adult freezing behaviour. These results are particularly intriguing in light of human literature that has suggested, similarly, that symptoms of anxiety and depression, as well as altered physiological responses to stress, during childhood could predict later adult depressive disorders, even at sub-threshold (non-clinical) levels (Garber, 2006; Shankman et al., 2009; Kovacs and Lopez-Duran, 2010). Thus, our model may provide important clues to some of the markers of altered developmental trajectory early in life that could contribute to later depressive symptoms, without a 'full blown' pediatric anxious/depressed phenotype.

The effects of EHC in our model were less dramatic than those often reported in later life environmental enrichment, with many of the impacts of EHC on behaviour appearing in juvenile animals, but disappearing by adulthood. Indeed, some of the effects

in juveniles were similar to the effects of PPS, supporting the idea of EE itself as a mild stressor (Connors et al., 2015), though importantly, EHC did prevent some of the adult effects of PPS on anxious (OFT-A freezing) and depressive (sucrose preference anhedonia) behaviours. As discussed previously, these results could suggest that the effects of PPS were more long-lasting than those of our EHC paradigm, as some studies suggest that the effects of EE fade over time (Rosenfeld and Weller, 2012), and that the (often independent) changes of EHC are potentially less 'powerful' than those of PPS (Darbra and Pallarès, 2011), based on the timing or type of exposure. Further research is required to determine if longer exposure, or exposure during a different developmental time period might have more potent and long lasting effects.

2.5. Further Studies

Taken together, the results of this study suggest that exposure to prenatal predator odour and postnatal environmental enrichment provides a novel, ethologically relevant model of prenatal stress, which may provide important insights into the developmental trajectory of anxiety/depressive behaviours. In particular, we see that some sex-specific changes in physiology and behaviour are already present at our juvenile time point (PND15-17), and that these changes could predict aspects of later neuropsychiatric vulnerabilities. Given this, we will begin to investigate some of the potential neural markers that could be disrupted by our PPS and EHC treatments at this age, as well as any potential sex differences in these systems. As the process of sexual differentiation is occurring during the same late prenatal and early postnatal period disrupted by our interventions, and is also potentially disrupted by prenatal stress exposure, we will also begin to delve into the potential role of organizational gonadal hormones in the normal

developmental expression of these markers at this same age. In Chapter 3 we will first investigate epigenetic changes in the PND15 brain caused by our PPS/EHC intervention, using IHC analysis of DNMT3a-ir density in a number of brain regions associated with neuropsychiatric disorders. In Chapter 4, we will then probe further into the role of sexual differentiation in organizing DNMT3a markers, and investigate the role of postnatal androgens or anti-androgens in establishing DNMT3a-ir patterns. In Chapter 5, we will examine the role of our PPS/EHC model in altering density of GAD67-ir cells in these same brain regions at this developmental time point, as GABA is a neurotransmitter previously linked to both DNMT3a-ir levels, as well as anxiety/depressive disorders and sexually dimorphic behaviour. Finally, in Chapter 6, we will then investigate the role of gonadal androgens in establishing the developmental patterns of GAD67-ir in these juvenile animals.

TABLE 2.1. COMPARISON OF LITTER CHARACTERISTICS ON THE DAY OF BIRTH BETWEEN DAMS EXPOSED TO PPS OR NC PRENATAL CONDITIONS.

LITTER CHARACTERISTICS AT BIRTH	NAÏVE CONTROL (NC)	PRENATAL PREDATOR STRESS (PPS)
LITTER SIZE	14.9 ± 0.74	12.5 ± 1.41
SEX RATIO (M:F)	0.48 ± 0.02	0.55 ± 0.04
BIRTH WEIGHT (G) – MALE	6.52 ± 0.12	6.1 ± 0.15*
BIRTH WEIGHT (G) – FEMALE	6.1 ± 0.1	5.6 ± 0.15*

Summary of litter characteristics on the day of birth comparing dams exposed to prenatal predator stress (PPS) to those in normal control conditions (NC). Data are presented as Mean ± SEM (Standard Error of the Mean). * indicates significant differences between PPS and NC.

TABLE 2.2. SUMMARY OF SIGNIFICANT CORRELATIONS BETWEEN DAM FECAL GLUCOCORTICOID (GC) LEVELS AND OFFSPRING BEHAVIOURS.

	GD15 PM	GD18 AM	AVG AM	AVG PM
OFT ACTIVITY				
J – Rearing		**-F: -SC**	**-F: -EHC**	
A - Rearing	**(+M: EHC)**	**-F** **-M: -SC**	**-F**	
OFT ANXIETY				
J - Center Time		**(-F: -EHC)**	**(-F: -EHC)**	
A - Center Time	**+M**	**-M (-SC)**	**(-M: -SC)**	
J - Freezing Time		**+F**		
A - Freezing Time		**(+F: +SC)** **(+M: +SC)**	**(-M: -EHC)**	**(-M: -EHC)**
FORCED SWIM TEST				
J - Immobility		**(+M: +SC)**	**+F** **(-M: EHC)**	**+F** **(-M: -EHC)**
A - Immobility	**(+F: EHC)**	**-F: -SC** **-M: -SC**		**(-M: -EHC)**
J - Latency		**(-M: -SC)**	**-F: -EHC**	**- F: -SC**
A - Latency	**(-F: -EHC)** **(-M: -EHC)**	**+F: +SC**	**+F**	
J - Climbing				
A - Climbing	**(+M : +SC)**	**-M** **(+F: +EHC)**	**+F: +EHC**	
SOCIAL INTERACTION				
Rough and Tumble Play				**-M: -EHC)** **(-F: -EHC)**
Exploration		**+M**	**-M:** **(+EHC)**	**-M:** **-SC/+EHC**
SUCROSE PREFERENCE				
Sucrose Preference %			**-F: -SC**	

Summary of significant correlations between prenatal dam fecal glucocorticoids (ng) and offspring behaviour. M = male, F = female, SC = standard cage, EHC = enhanced housing condition, J = Juvenile, A = Adult. +/- indicates direction of effects, ( ) indicates that effects are only present in the indicated postnatal treatment group.

TABLE 2.3. SUMMARY OF SIGNIFICANT CORRELATIONS BETWEEN JUVENILE AND ADULT OFFSPRING BEHAVIOURS.

ADULT / Juvenile	OFT Rearing	OFT Freezing	SI Exploration	FST Swimming	FST Climbing	FST Latency to First Immobility	FST Immobility	Sucrose %
OFT Rearing				-M	-F	-M	+F	
OFT Grooming						+M		+M
OFT Freezing	-F			+F		+F	-F -M	
OFT Center Time					-F			
FST Immobility		-F +M	-F					
FST Latency to Immobility		-M						
Social Interaction Test: Rough and Tumble Play				-M				-M
Social Interation Test: Exploration	+F			+M				

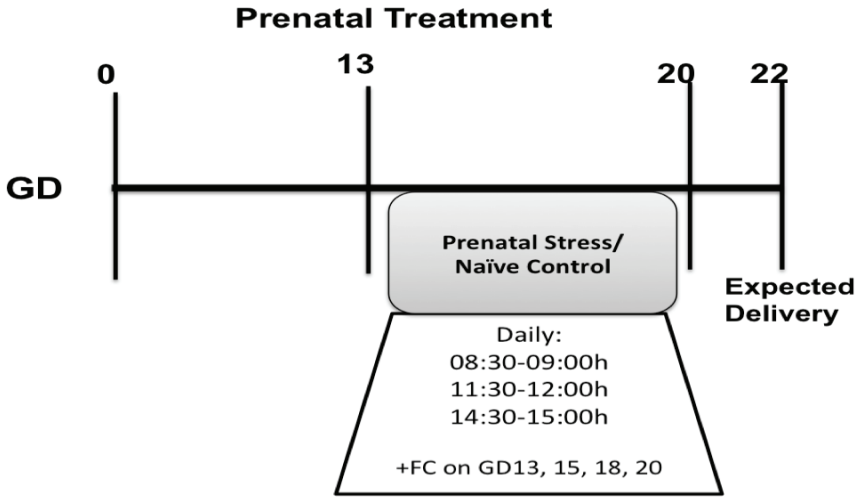
Summary of significant correlations between juvenile and adult and offspring behaviour. +/- indicates direction of effects, M = male, F = female, SC = standard cage, EHC = enhanced housing condition.

Table 2.4. Summary of significant effects of treatment on juvenile and adult offspring behaviours.

	Effects of PPS	Effects of EHC	Effects of PPS + EHC
OFT ACTIVITY			
Juvenile Line Crosses	↓ M	↓ M ↓ F	– M
Adult Line Crosses		↓ M	– M
Juvenile Rearing Time			
Adult Rearing Time		↓ F	
OFT CENTER TIME			
Juvenile Center Time			
Adult Center Time	↓M		
Juvenile Freezing	↑ F		
Adult Freezing	↑ M ↑ F	↓ F	– M – F
FORCED SWIM TEST			
Juvenile Immobility		↓F	
Adult Immobility			
Juvenile Latency to Immobility	↓ M ↓ F	↑ F	– F
Adult Latency to Immobility			
Juvenile Climbing			
Adult Climbing	↓ M		
SOCIAL INTERACTION TEST			
Rough and Tumble Play	↓M		
Exploration			↑ M ↑ F
Social Rest	↑ M		– M
Self-Grooming	↑ F		
SUCROSE PREFERENCE TEST			
Sucrose Preference %	↓F		– F

Summary of significant group effects of treatment on offspring behaviour by prenatal predator stress (PPS) alone, enhanced housing condition (EHC) alone, and cases where PPS/EHC resulted in a different effect where either alone. ↑/↓ indicates direction of effects, and – indicates cases where PPS+EHC rescued/prevented effects of either alone. M = male, F = female.

A.



B.

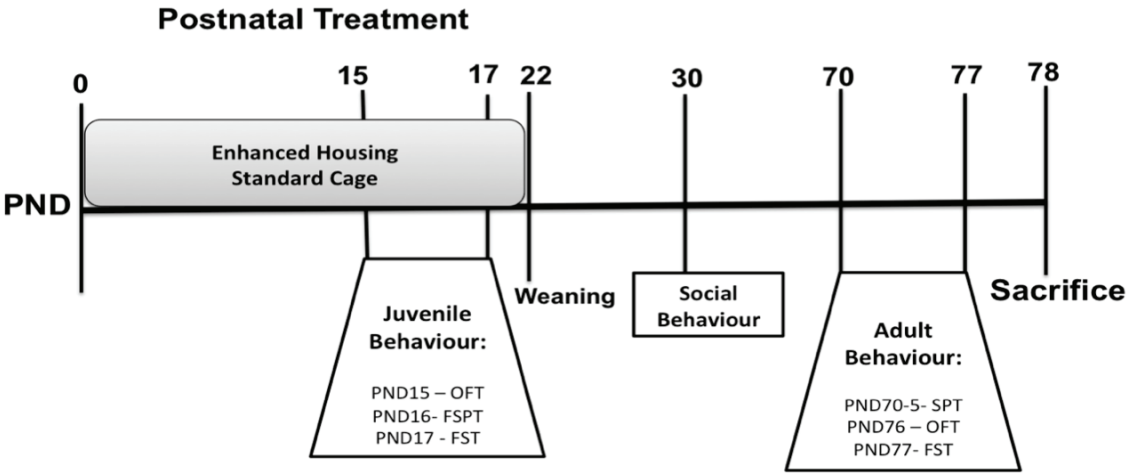


FIGURE 2.1. TIMELINE OF PRENATAL (A) AND POSTNATAL (B) TREATMENT PROCEDURES.

A. Dams designated to receive prenatal psychological stress (PPS) were exposed three times daily to a predator threat stressor between gestational day (GD) 13 and GD20, along with fecal collection (FC) on GD 13, 15, 18, 20. **B**. On the day of birth, postnatal day (PND) 0, dams and pups were transferred to either an Enhanced Housing Cage (EHC), or a clean Standard Cage (SC). Behavioural tests consisted of juvenile and adult Open Field Test (OFT), a juvenile Forced Swim Pretest (FSPT) and juvenile and adult Forced Swim Test (FST), a peripubertal social interaction (SI) test, and an adult Sucrose Preference Test (SPT) before sacrifice.

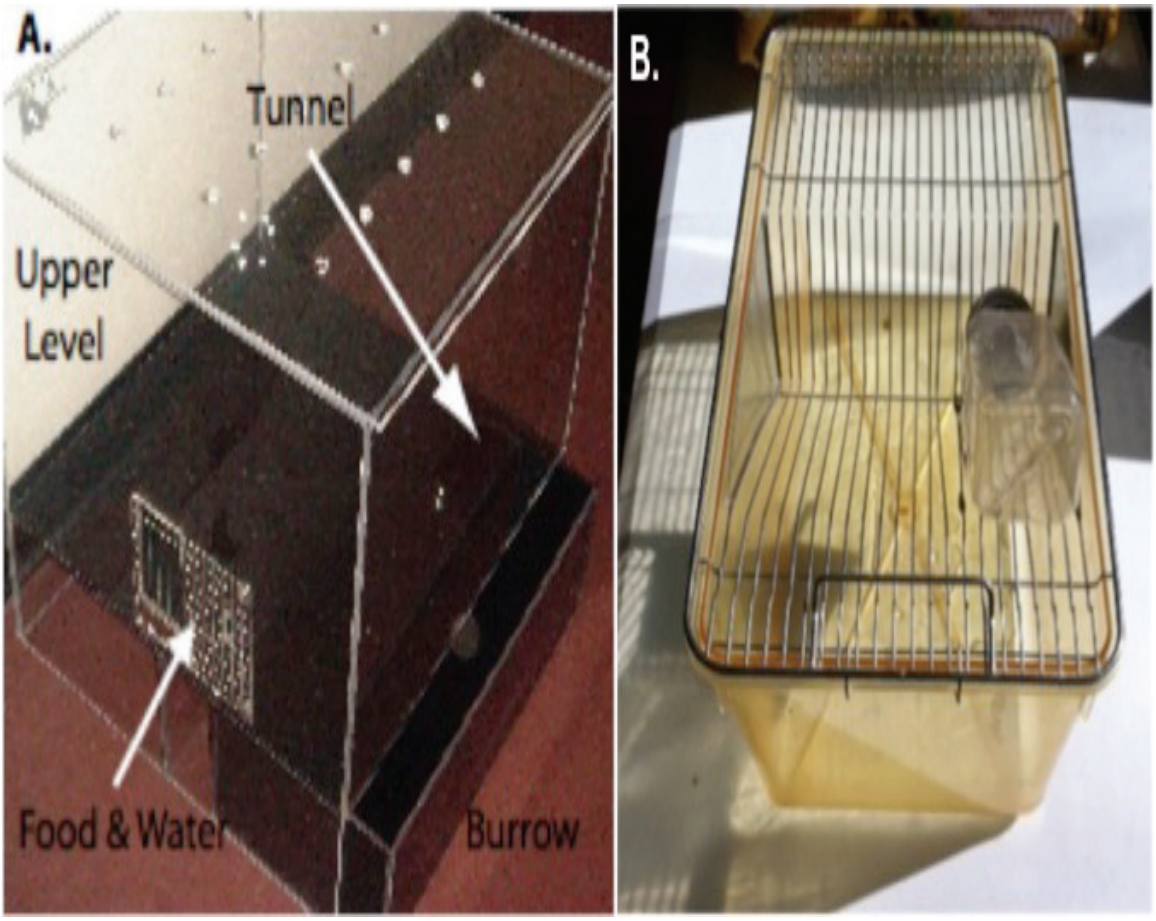


Figure 2.2. Schematic of the (A) enhanced housing condition (EHC) and (B) standard cage (SC) home cage.

A. Enhanced Housing Condition (EHC) home cage, containing a 'burrow' section (contained within a drawer that moves out to facilitate cleaning) and an 'upper' section (containing food and water). The two sections are connected by a tunnel (visible in the 'upper' section of the schematic). **B.** Representative image of a standard home cage (SC).

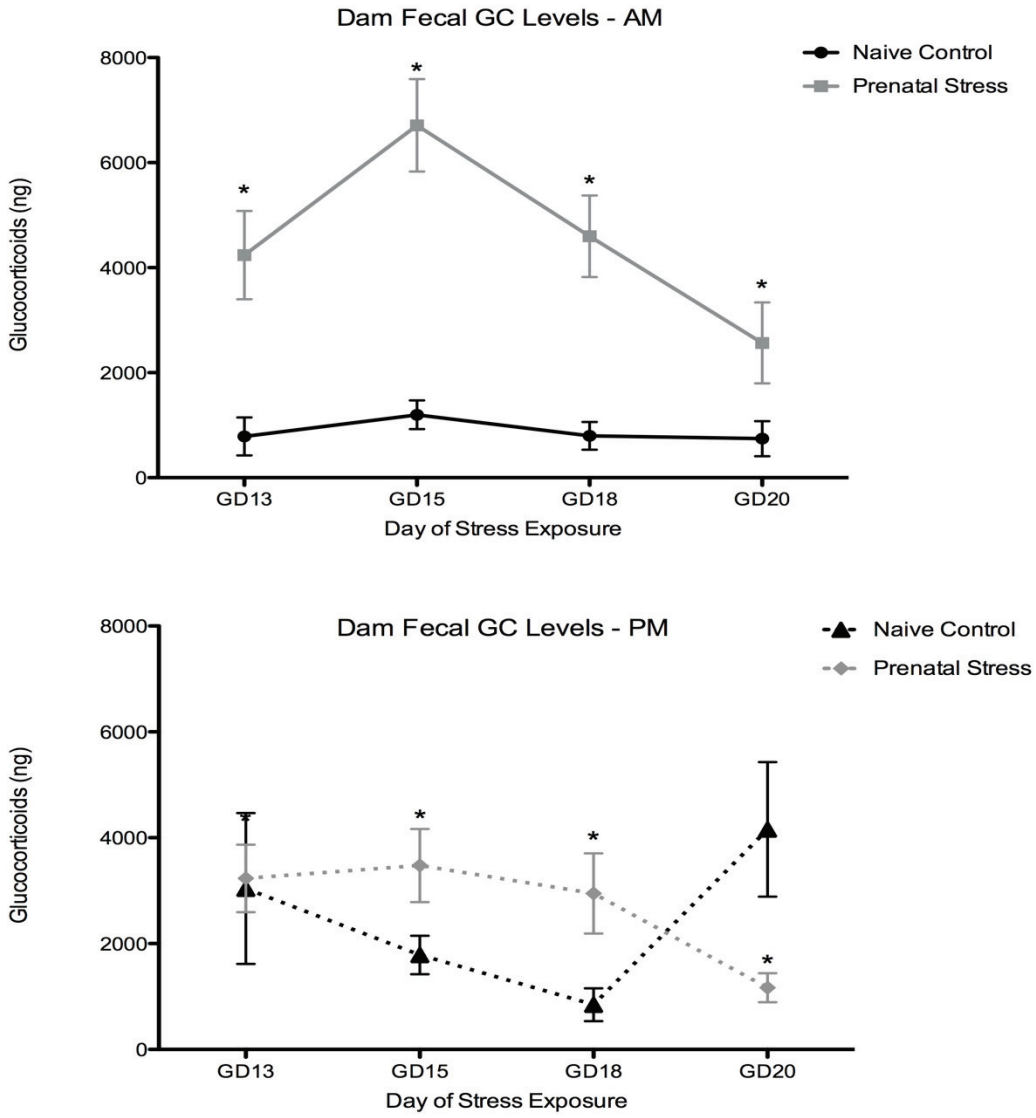


FIGURE 2.3. TOTAL GLUCOCORTICOIDS (GCS) MEASURED IN FECAL SAMPLES COLLECTED FROM DAMS RANDOMLY ASSIGNED TO A PRENATAL NAIVE CONTROL (NC) OR PRENATAL STRESS (PPS) CONDITION.

Total glucocorticoids (GCs) measured in fecal samples collected from dams randomly assigned to a prenatal naive control (NC) or prenatal predator stress (PPS) condition. Fecal samples were collected following the third and final daily cat exposure session on days 1, 3, 6, and 8 of the gestational stress period (gestational days 13–20) either **A.** during the morning stressor or **B.** during the afternoon stress exposure. * Significantly different from NC for that day, $p < 0.05$.

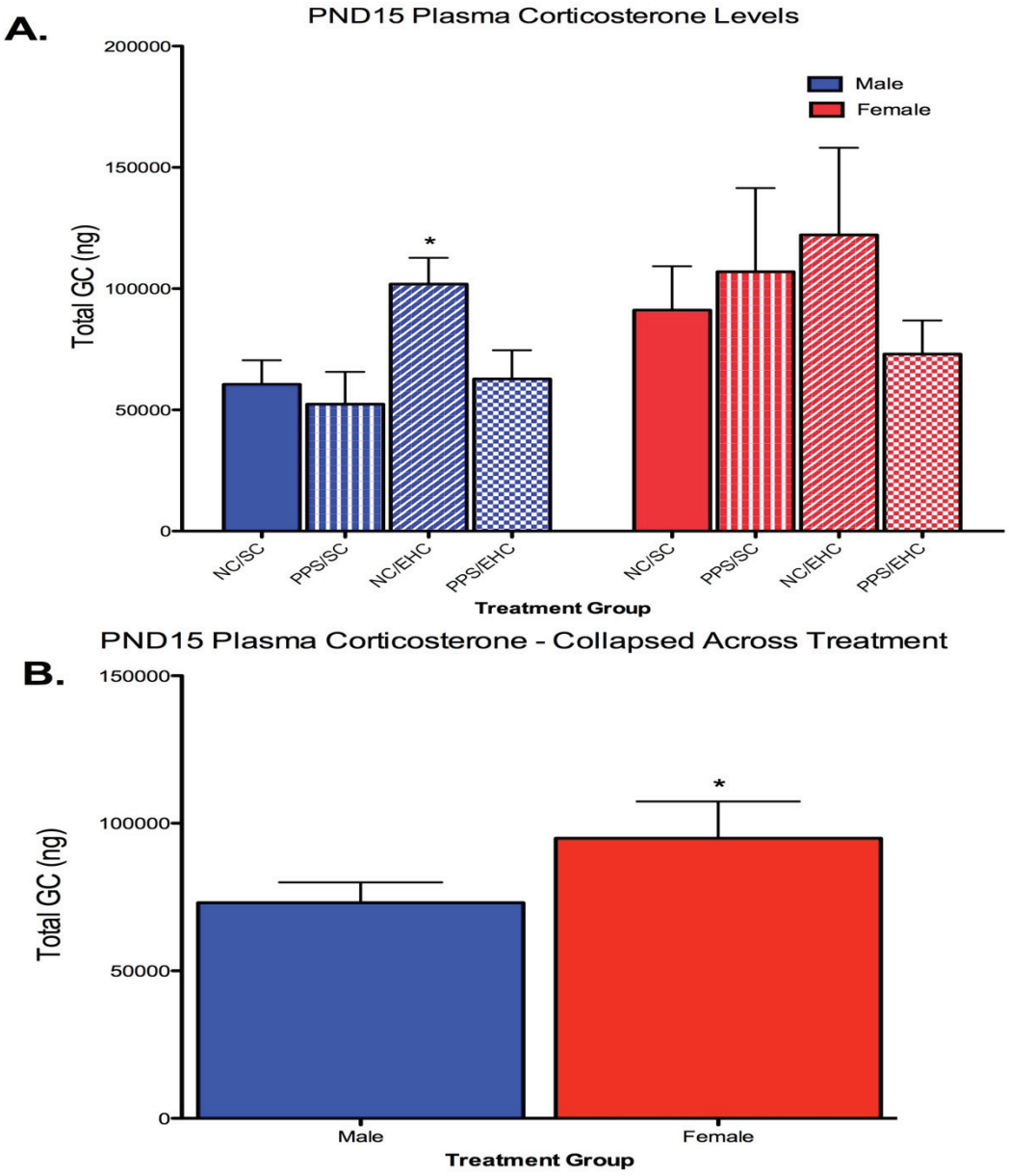


FIGURE 2.4. TOTAL GLUCOCORTICOIDS (GCS) MEASURED IN PLASMA TRUNK BLOOD SAMPLES COLLECTED FROM PND15 MALE AND FEMALE OFFSPRING FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total glucocorticoids (GCs) measured in plasma samples collected from trunk blood plasma samples in male and female pups sacrificed on PND15, from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** All samples separated by treatment group, * Significantly different from NC/SC, $p < 0.05$. **B.** Samples collapsed across treatment to illustrate a main effect of sex. * significantly different from males on PND15, $p < 0.05$.

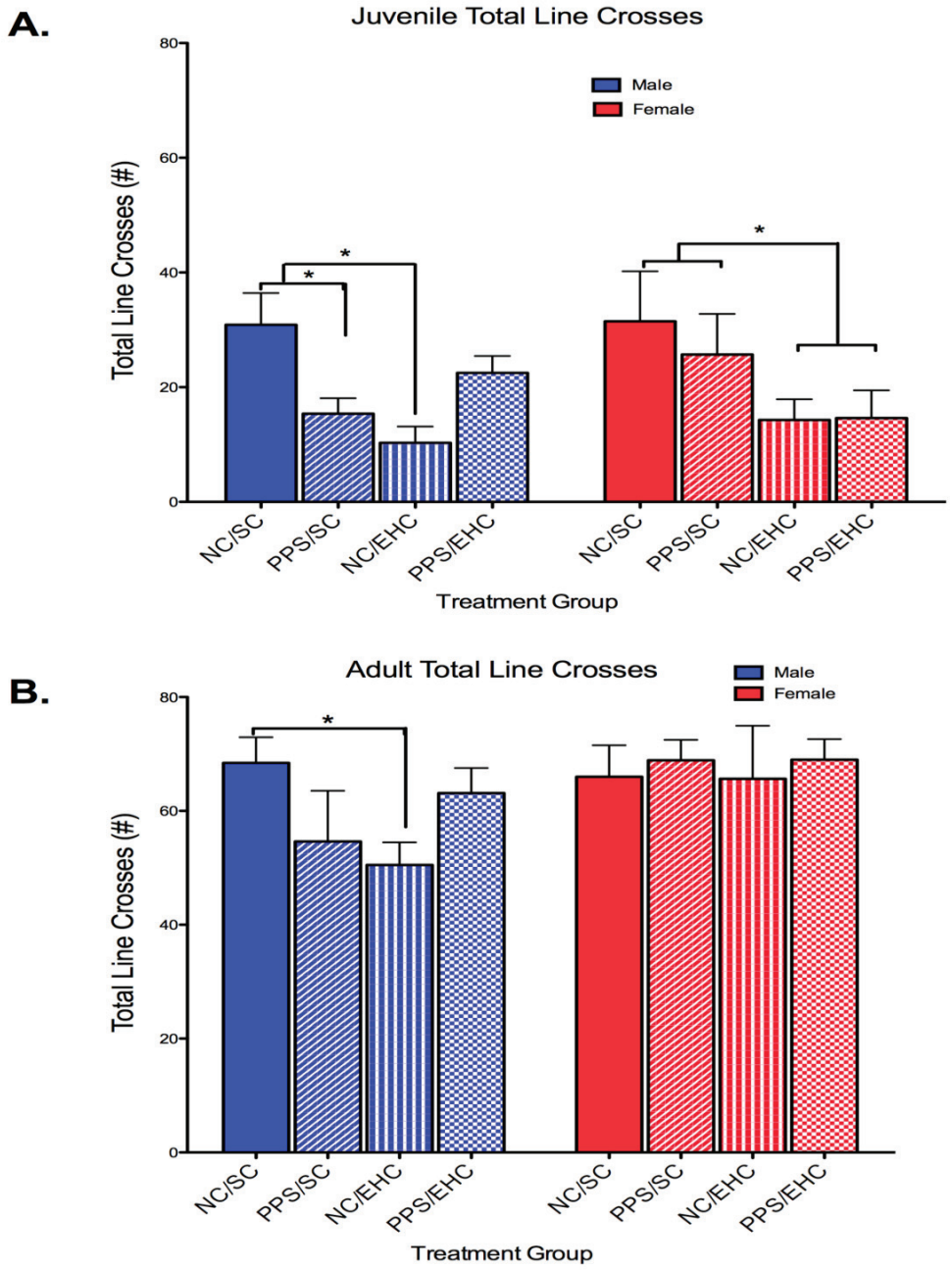


FIGURE 2.5. TOTAL LINE CROSSED MEASURED ON JUVENILE AND ADULT OPEN FIELD TESTS IN MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total number of line crosses in the Open Field Test (OFT) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males and females on PND15, or **B.** Adult males and females on PND76. * significantly different from same-sex NC/SC at each age, $p < 0.05$.

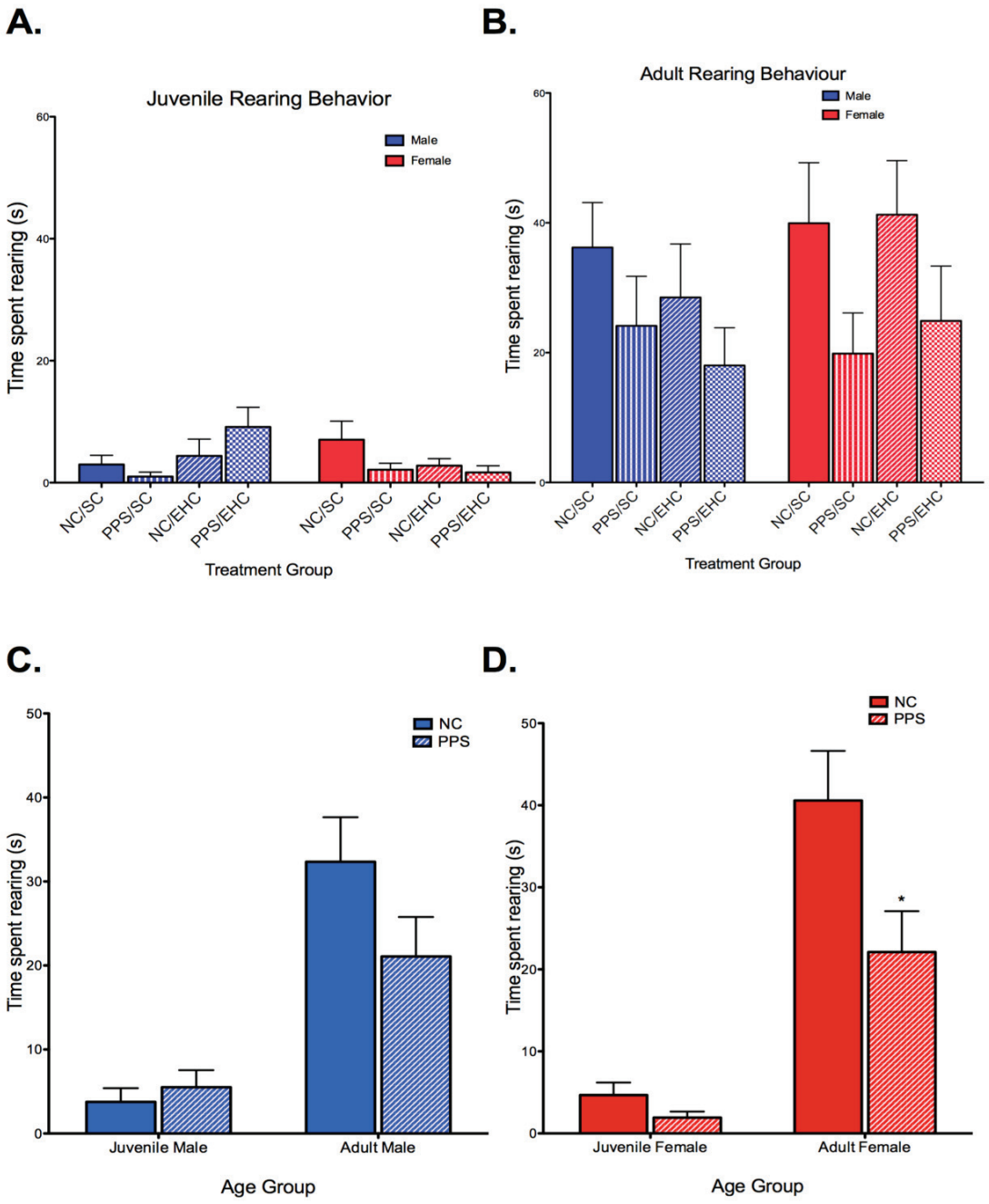


FIGURE 2.6. TOTAL TIME SPENT REARING IN JUVENILE AND ADULT OPEN FIELD TESTS ON MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total time spent rearing (s) in the Open Field Test (OFT) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males and females on PND15, or **B.** Adult males and females on PND76. **C.** Male subjects collapsed across postnatal treatment groups **D.** Female subjects collapsed across postnatal treatment group to illustrate an effect of PPS in adulthood. *significantly different from adult NC females, $p < 0.05$.

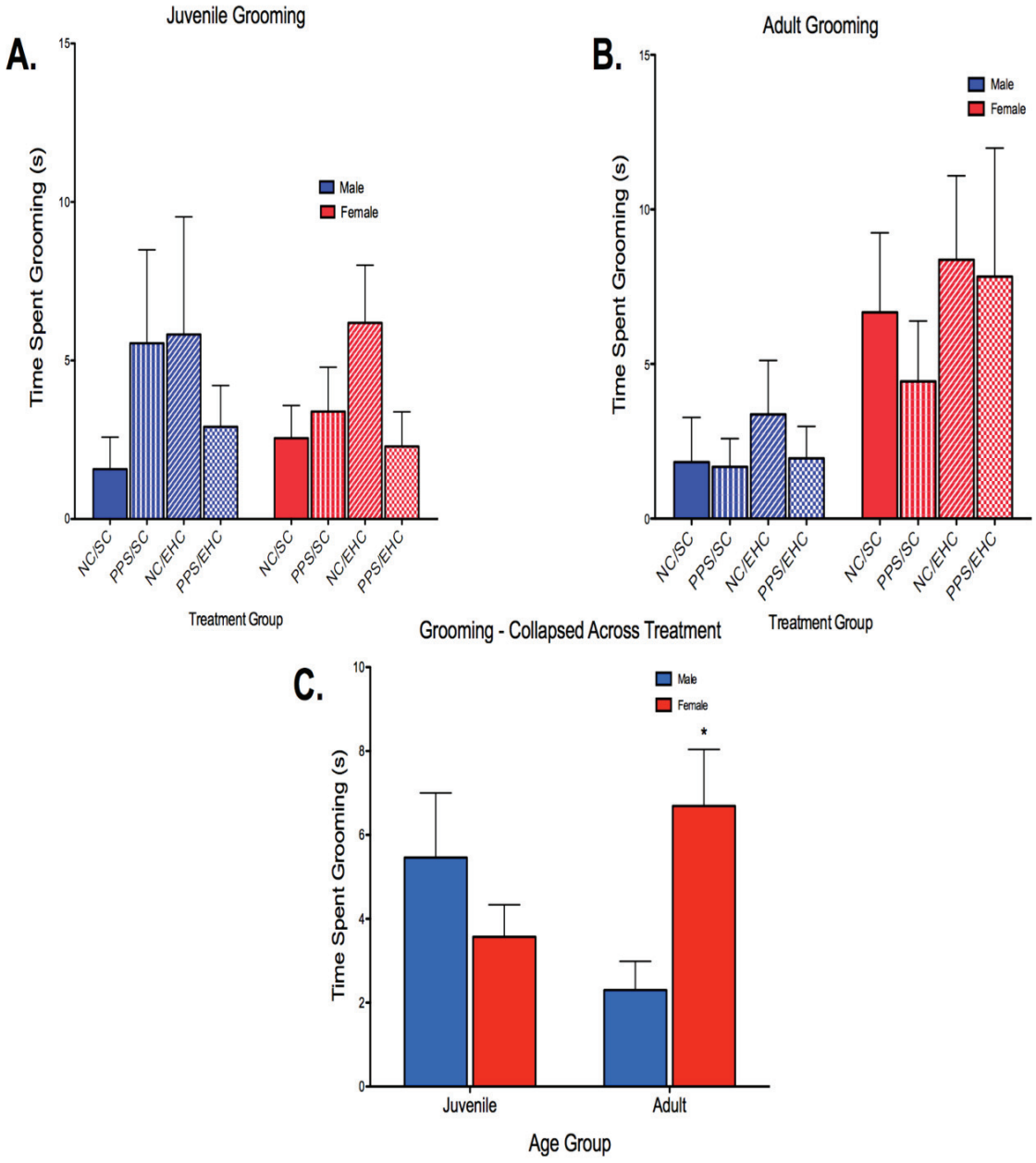


FIGURE 2.7. TOTAL TIME SPENT GROOMING ON JUVENILE AND ADULT OPEN FIELD TESTS IN MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent grooming in the Open Field Test (OFT) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males and females on PND15, or **B.** Adult males and females on PND76. **C.** Subjects collapsed across prenatal and postnatal treatment groups to illustrate an effect of sex in adulthood. * significantly different from adult males, $p < 0.05$.

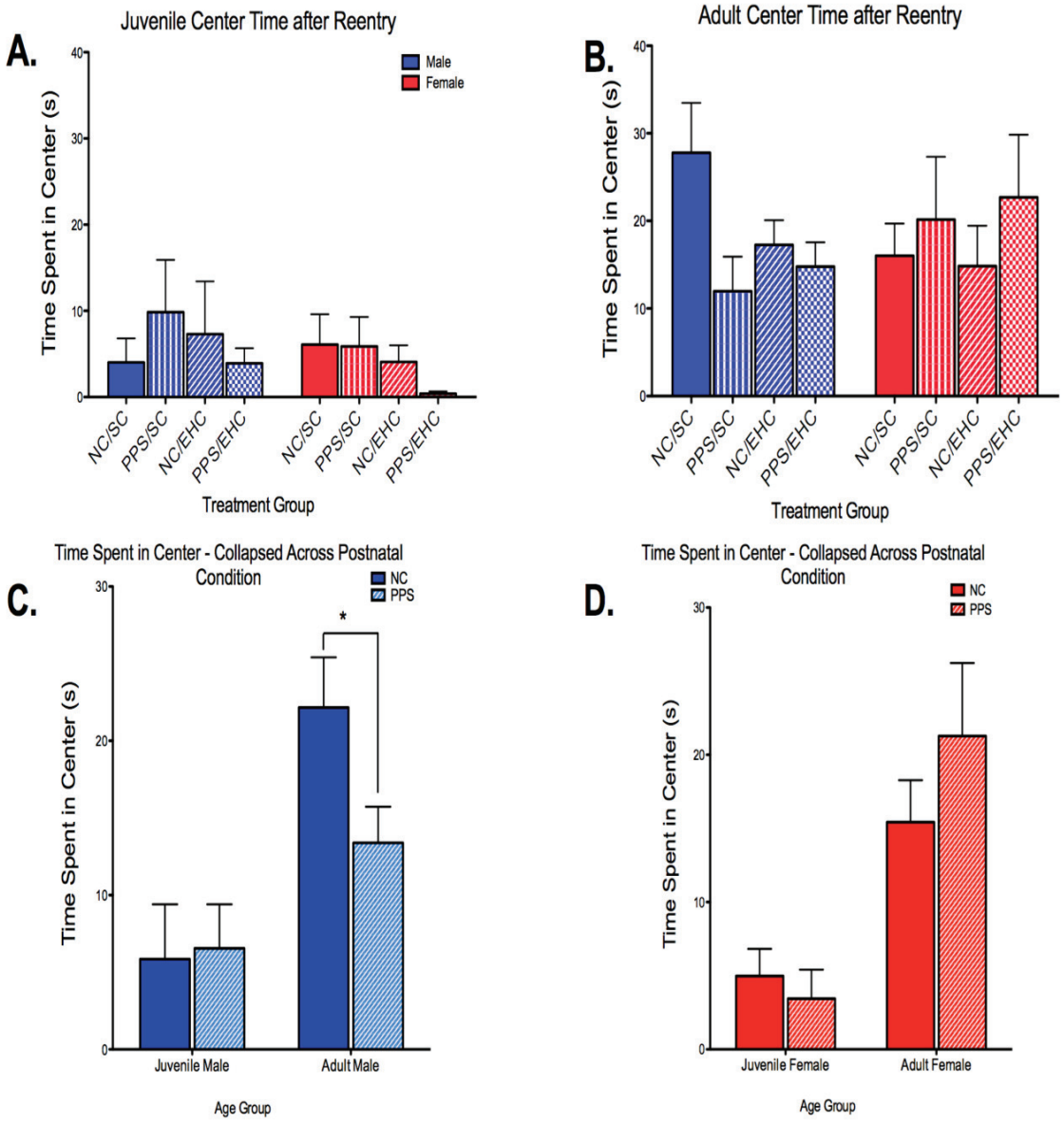


FIGURE 2.8. TOTAL TIME SPENT IN THE CENTER OF AN OPEN FIELD TEST IN JUVENILE AND ADULT MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent in the center squares of the Open Field Test (OFT) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males and females on PND15, or **B.** Adult males and females on PND76. **C.** Male subjects collapsed across postnatal treatment groups to illustrate an effect of sex in adulthood. **D.** Female subjects collapsed across postnatal treatment group. *significantly different from adult NC males, $p < 0.05$.

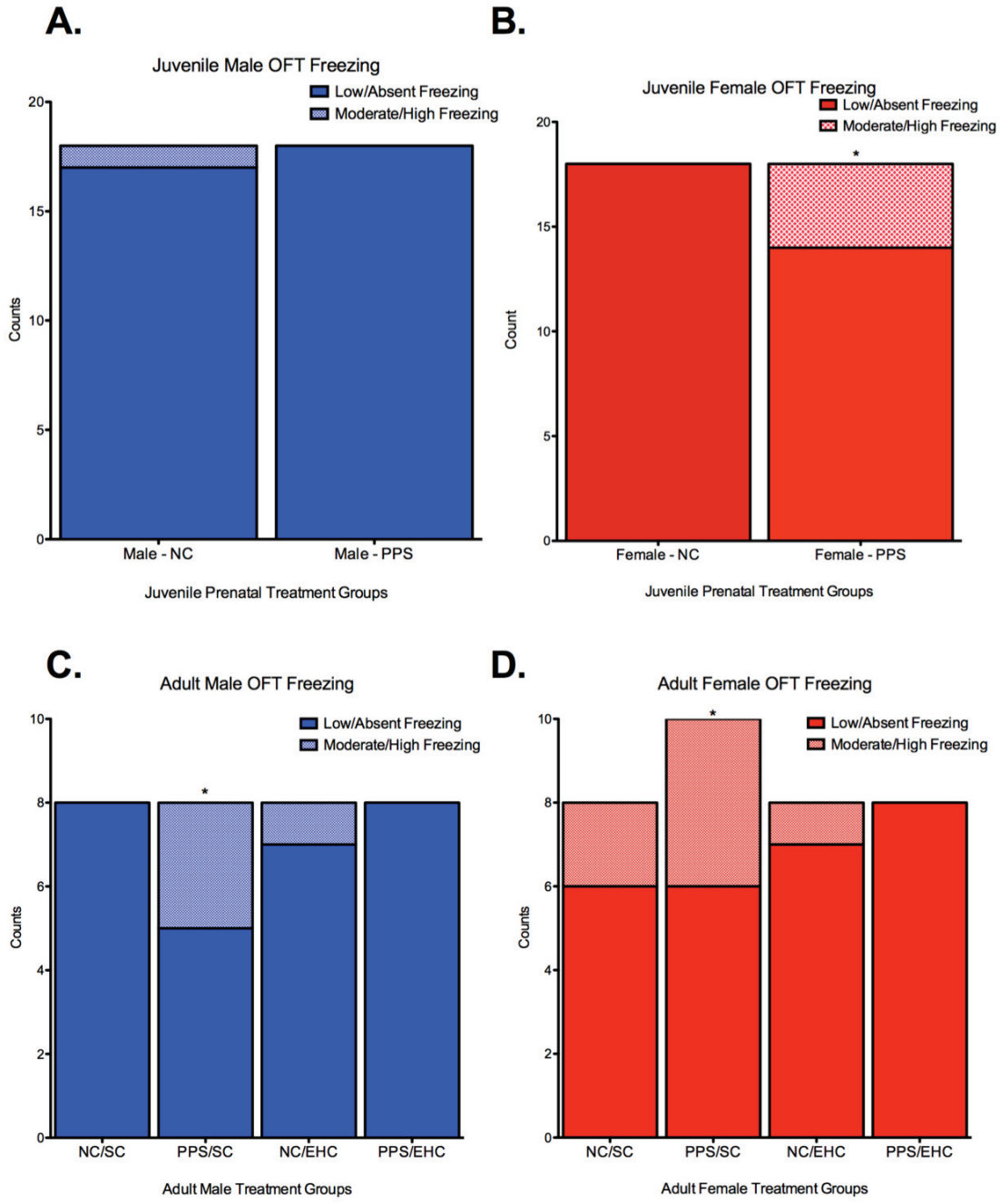


FIGURE 2.9. TOTAL FREQUENCY COUNTS OF ANIMALS ENGAGED IN LOW/ABSENT OR MODERATE/HIGH FREEZING TIME IN JUVENILE AND ADULT OPEN FIELD TESTS IN MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.
Frequency counts of offspring engaged in low/absent or moderate/high time spent freezing in the Open Field Test (OFT) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males on PND15 **B.** Juvenile females on PND15. **C.** Adult males on PND76. **D.** Adult females on PND76. * significant difference from frequency in other treatment conditions, $p < 0.05$.

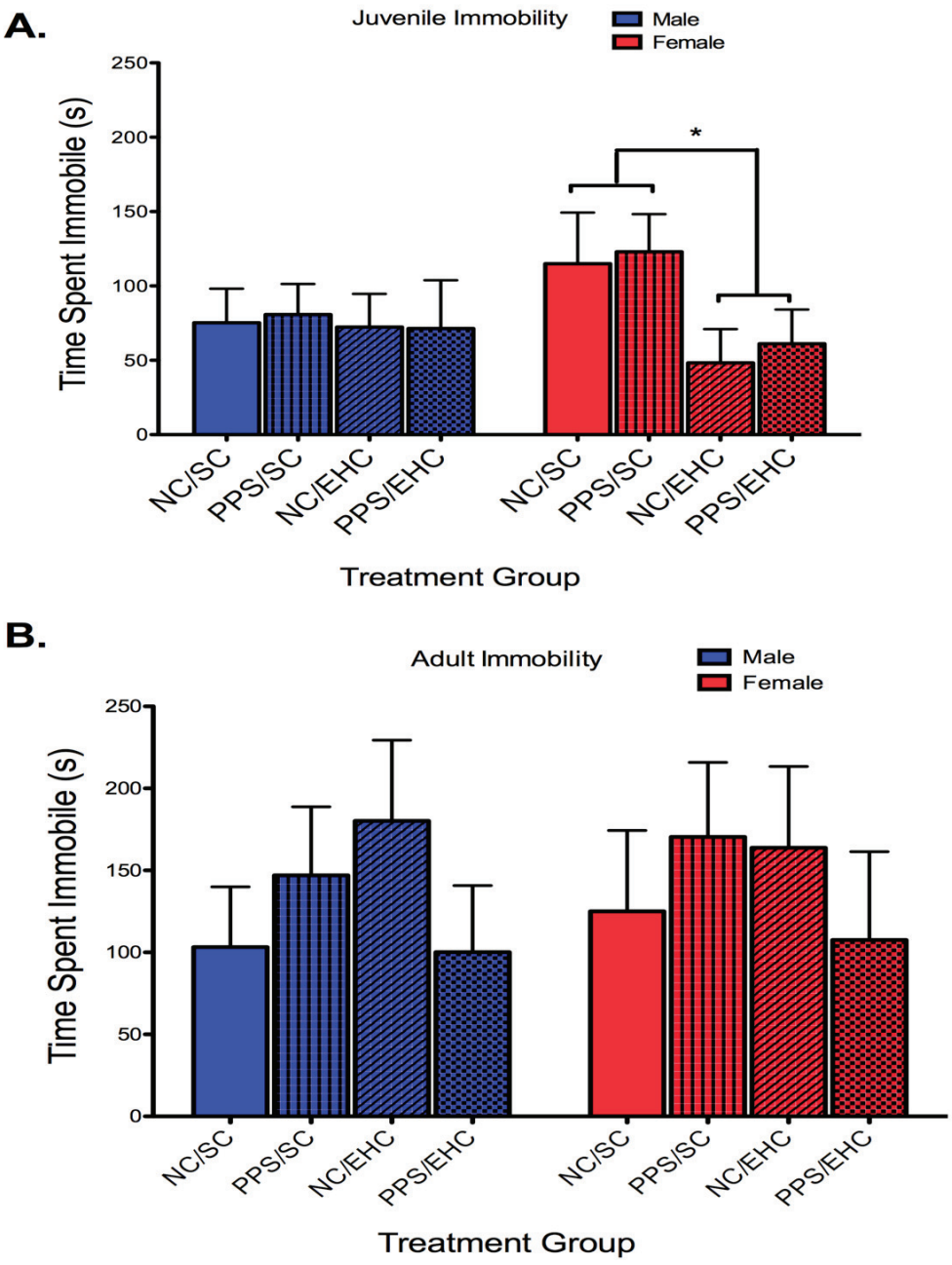


FIGURE 2.10. TOTAL TIME SPENT IMMOBILE IN A FORCED SWIM TEST IN JUVENILE AND ADULT MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent immobile in the Forced Swim Test (FST) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. A. Juvenile males and females on PND15, or B. Adult males and females on PND77. * significant difference from NC, $p < 0.05$.

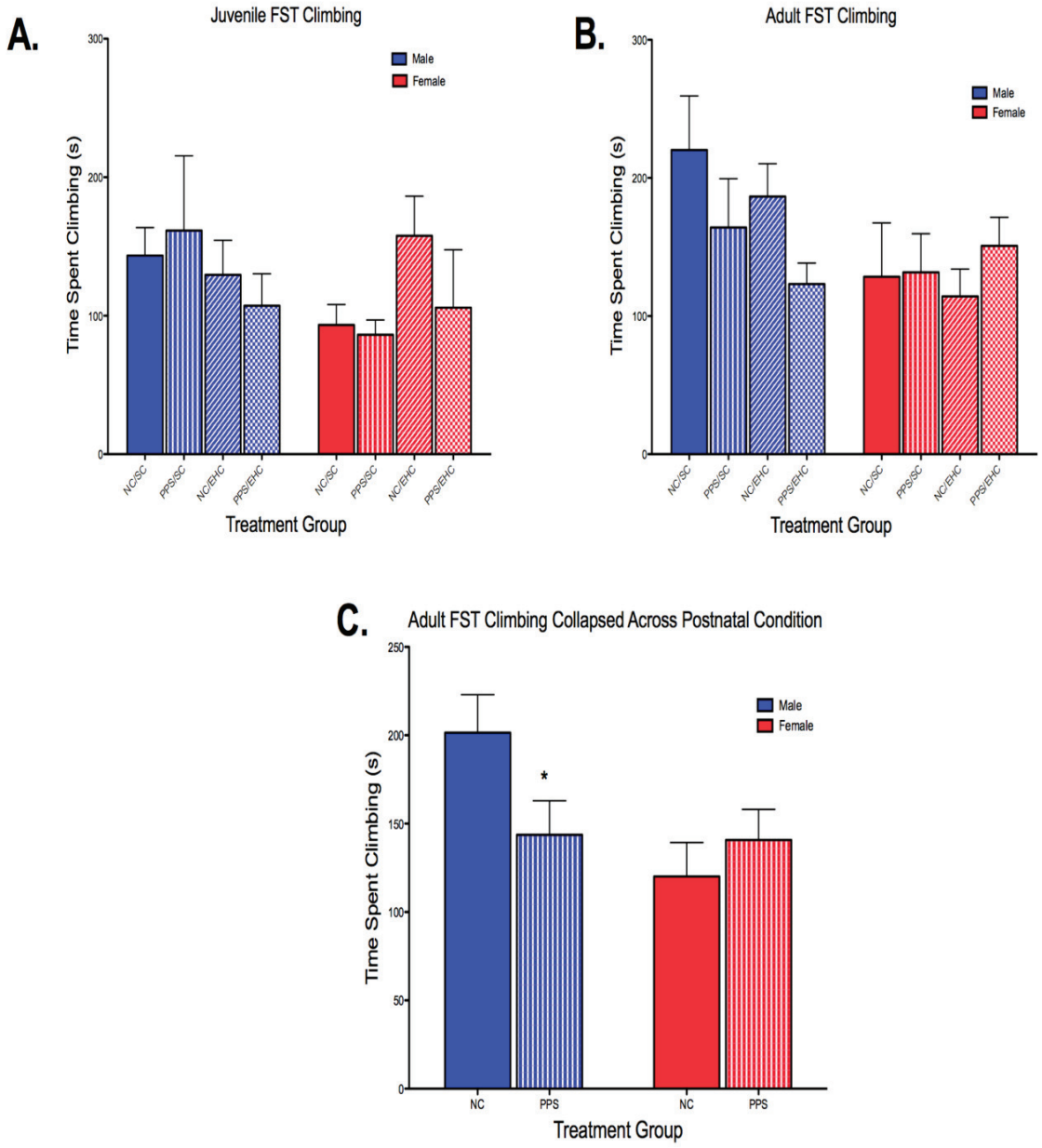


FIGURE 2.11. TOTAL TIME SPENT CLIMBING IN A FORCED SWIM TEST IN JUVENILE AND ADULT MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent climbing in the Forced Swim Test (FST) in rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Juvenile males and females on PND15 **B.** Adult males and females on PND77. **C.** Climbing in adult males and femalesn collapsed across postnatal treatment to illustrate an effet of PPS. * significant difference from NC males, $p < 0.05$.

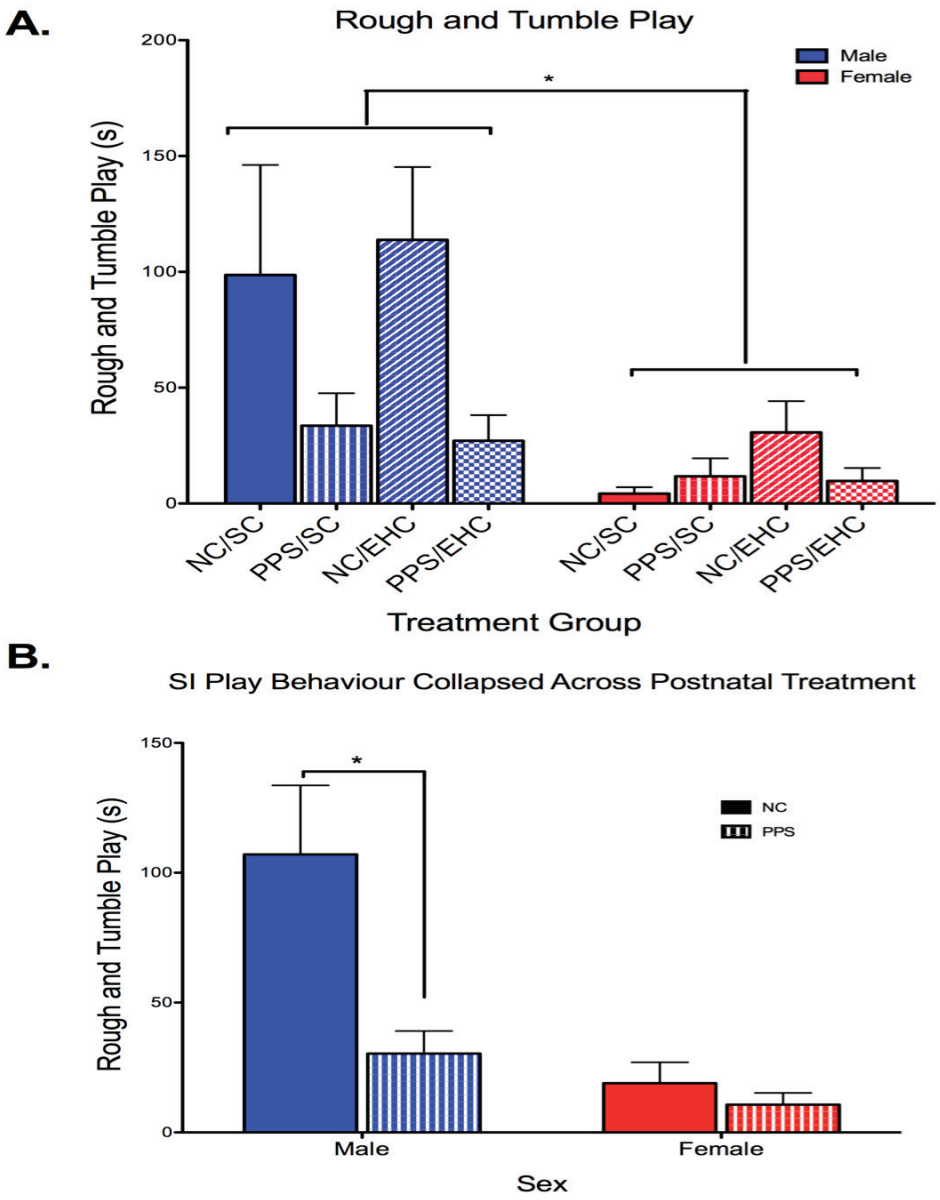


FIGURE 2.12. TOTAL TIME SPENT IN ROUGH AND TUMBLE PLAY ON A PERI-PUBERTAL SOCIAL INTERACTION TEST IN MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent in various play behaviours during a peri-pubertal (PND30) social interaction (SI) test in male and female rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Time spent in rough and tumble play behaviour for males and females in each treatment group, * significantly different from males, $p < 0.05$.
B. Subjects collapsed across postnatal treatment group to illustrate an effect of PPS on male rough and tumble play * significantly different from NC males, $p < 0.05$.

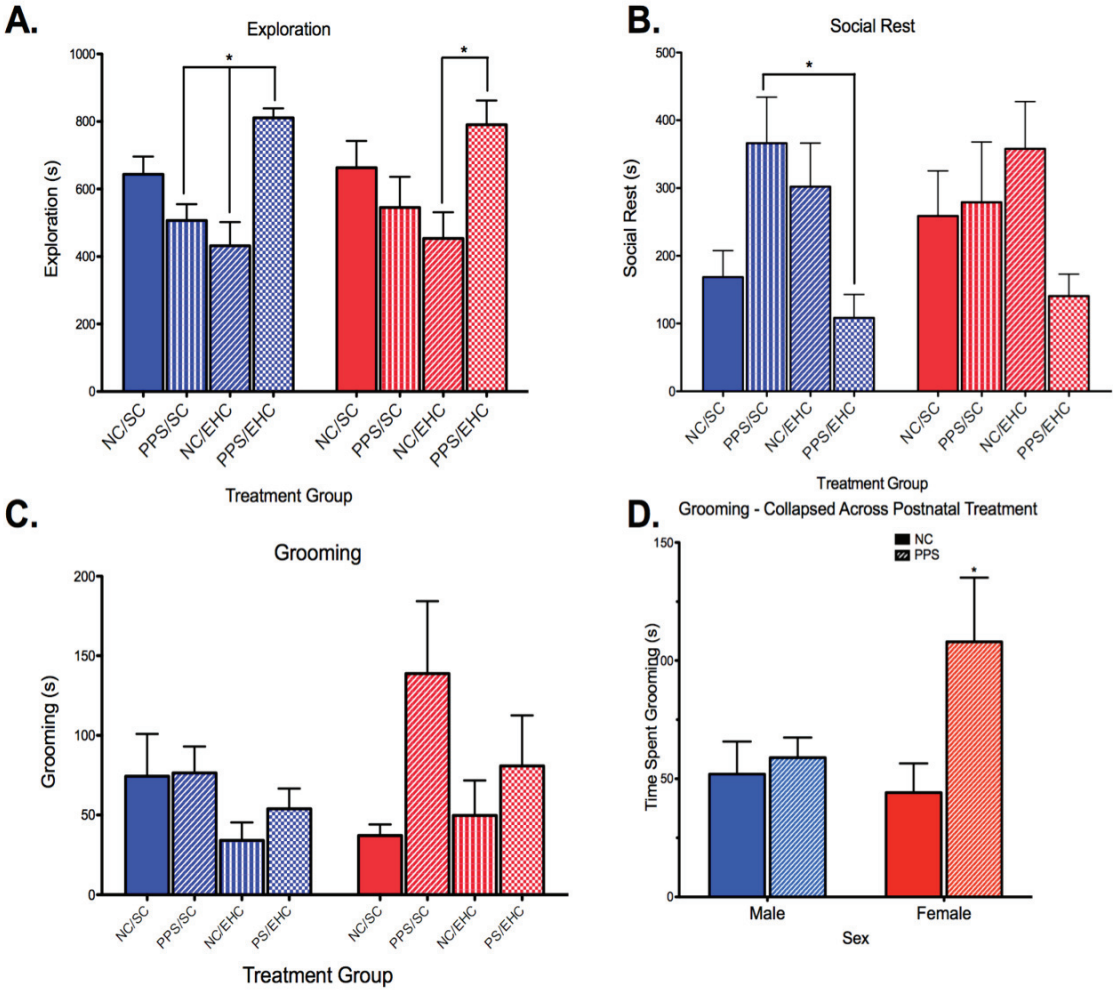


FIGURE 2.13. TOTAL TIME SPENT IN SOCIAL BEHAVIOURS ON A PERI-PUBERTAL SOCIAL INTERACTION TEST IN MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Total amount of time spent in various play behaviours during a peri-pubertal (PND30) social interaction (SI) test in male and female rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. **A.** Time spent in non-social exploration of the environment for males and females in each treatment group. **B.** Time spent in social rest for males and females in each treatment group **C.** Time spent in self-grooming for males and females in each treatment group **D.** Subjects collapsed across postnatal treatment group to illustrate an effect of PPS in females. * significant difference from indicated groups, $p < 0.05$.

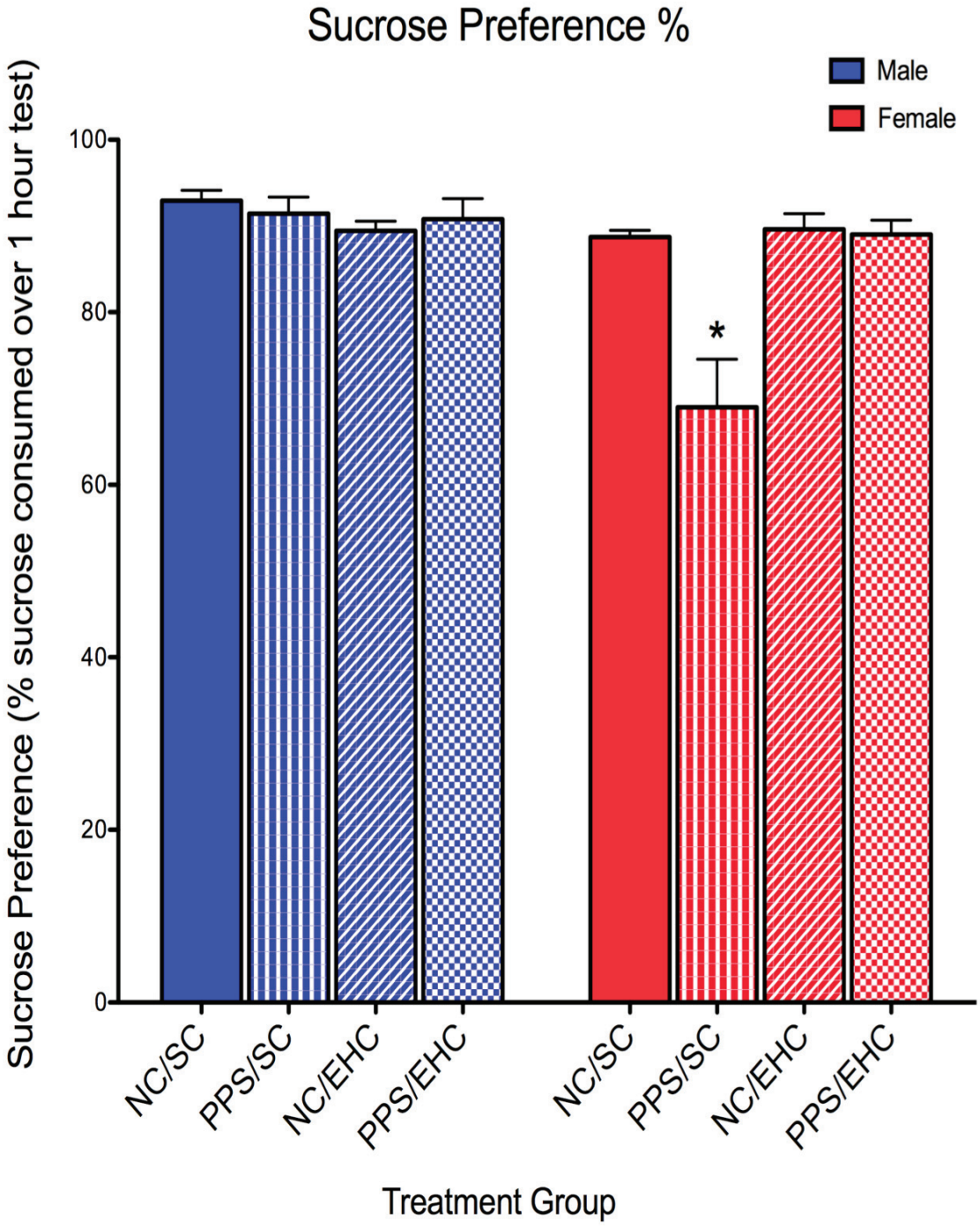


FIGURE 2.14. SUCROSE PREFERENCE (% SUCROSE) IN ADULT MALES AND FEMALES FOLLOWING PRENATAL AND POSTNATAL INTERVENTIONS.

Sucrose preference (% of sucrose consumed/ total fluid) in a two-bottle choice paradigm on adult (PND75) male and female rats from a prenatal naive control (NC) or prenatal predator stress (PPS) condition, with a postnatal standard cage (SC) or enhanced housing condition (EHC) intervention. * significantly different from same-sex NC/SC, $p < 0.05$.

CHAPTER 3 – EPIGENETIC CHANGES IN THE JUVENILE BRAIN FOLLOWING PRENATAL PREDATOR EXPOSURE AND EARLY LIFE HOME CAGE ENHANCEMENT

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3.1. – Introduction

Vulnerability to a number of neuropsychiatric disorders, including anxiety and depression, is strongly influenced by environmental factors. Epigenetic mechanisms are increasingly being investigated as important targets of these factors involved in shaping the brain's response to the environment, both in adulthood and in early life. For example, epigenetic changes underlie some neural and behavioural responses to stress, have been implicated in the vulnerability to neuropsychiatric disorders, and are also involved in processes underlying sexual differentiation of the brain (reviewed in Jessen and Auger, 2011; Billack et al., 2012; Baker-Andresen et al., 2013; Hodes, 2013; Bock et al., 2015; Mccarthy and Nugent, 2015; Mcgowan and Roth, 2015). Thus, it is reasonable to expect that there are sex- and region- specific epigenetic changes in the brain, both during typical development and in response to early life stress, which could provide clues to the underlying mechanisms of later vulnerability to anxiety and depression.

Epigenetic events alter the expression of genes in a number of complex ways (Mcgowan and Roth, 2015). Genes can be suppressed when promoters or other regulatory sequences are methylated. Specifically when a methyl group is transferred to a C-5 position of cytosine, this causes changes in chromatin structure that prevent transcription, as methylated cytosines bind repressor proteins, including the methyl-binding domain protein (MeCP2) and histone deacetylases (HDACs; Moore et al., 2013)). Methylation of a gene usually correlates with lower levels of the gene products, however, the relationship between methylation and transcription can be more complex depending on where on the gene sequence cytosines are bound (Mcgowan and Roth, 2015). A family of enzymes including the DNA methyltransferases (i.e. DNMT1, 3a, 3b, 3L) can carry out these

methylation events. DNMT1 is considered particularly important in maintaining patterns of methylation as it shows a preference for hemi-methylated sites, and may also contribute to some de novo methylation (Cheng and Blumenthal, 2008). DNMT3a and 3b are considered important for de novo methylation, where DNMT3b is thought to primarily be expressed early on in neuroprogenitor cells, while DNMT3a is expressed in post mitotic newly differentiated cells, and may play a larger role in cellular maturation and differentiation (Feng et al., 2005).

Epigenetic changes are found clinically in both patients with depression and following treatment with antidepressants, though the direction of effects is highly dependent on the tissue being studied and which promoters are being methylated (reviewed in Menke and Binder, 2014). Both polymorphisms in the DNMT3a and DNMT3B gene, as well as increased overall global levels of methylation, have been associated with suicide attempts in psychiatric patients (Maciag et al., 2010; Murphy et al., 2012). Similarly, decreased peripheral (white blood cell) levels of DNMT1 and DNMT3b have been found in patients currently experiencing a depressive episode, but not those in remission (Higuchi et al., 2011), and individuals with a life history of depression exhibit different methylation patterns in a number of genes associated with depression (Uddin et al., 2011). Changes in DNMT1 and DNMT3b have also been associated with posttraumatic stress disorder (PTSD), where lower levels of DNMT3b prior to significant stress are associated with a greater risk of post-traumatic stress disorder (PTSD) and worse PTSD symptoms, and higher post-stress levels of DNMT1 were found in PTSD cases compared to controls (Sipahi et al., 2014). In animal models, drugs which function as antidepressants or mood stabilizers can decrease levels of

DNMT1 and DNMT3a in specific brain regions, and blocking methylation may mimic some effects of antidepressants (Melas et al., 2012; Zimmermann et al., 2012).

In both human and animal models, exposure to high levels of stress, either in early life or adulthood, are significant risk factors for vulnerability to neuropsychiatric disorders (reviewed in Duman, 2010; Schmidt, 2011; Morley-Fletcher et al., 2013; Glover, 2014; Pizzagalli, 2014). In rodents, levels of DNMT3a are also affected by chronic stress in adulthood: in male rats chronic social defeat stress reduced levels of DNMT3a in the dentate gyrus, which correlated with greater depressive behaviours in defeated males (Hammels et al., 2014). In the nucleus accumbens, however, increased levels of DNMT3a mRNA are found following chronic stress in adult males, and overexpressing DNMT3a in the nucleus accumbens increased depressive behaviours in the FST (Laplant et al., 2010), suggesting the relationship between DNMT3a, stress, and depression may be region dependent. One recent study has found that chronic mild stress has both region, and sex-specific effects on global DNA methylation levels in brain regions including the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST) and central amygdala (CeA; Sterrenburg et al., 2011), suggesting that, in addition to regional differences, levels of DNA methylation in response to stress may also vary by sex. Given that there are sex differences in both the response to stress and in vulnerability to anxiety/depression, changes in DNMT3a may be an important mechanism underlying both processes, though there is still a relative paucity of research examining sex differences in either stress or depression.

Changes in DNMTs may be particularly important in maintaining the long-term impact of early-life events. Epigenetic changes represent an important mechanism by

which early life events can result in long-term, and even trans-generational, changes in the brain and behaviour (reviewed in Bale, 2011; Jessen and Auger, 2011; Szyf, 2011; Billack et al., 2012; Hodes, 2013; Laviola and Macrì, 2013; Bock et al., 2015; Cartier et al., 2015; Mcgowan and Roth, 2015). A plethora of studies exist in both human and animal research which suggest that early life stressors (predator threat, prenatal restraint, maternal separation, maternal depression, etc.) can increase the vulnerability to later mental health problems, and permanently alter a number of parameters of stress responding in sexually dimorphic ways. A recent human study has demonstrated that stress in early life significantly alters patterns of DNA methylation at a number of CpG sites in adolescence, and that these changes are sex-specific (Essex et al., 2013). In the animal literature, early prenatal stress has been shown to have sex-specific effects on methylation, increasing placental levels of DNMT1 in male pups (Mueller and Bale, 2008), and decreasing methylation of promoters linked to corticotrophin-releasing factor (CRF) in the amygdala, while increasing methylation of glucocorticoid receptor promoters in the hippocampus in male offspring (Mueller and Bale, 2008, reviewed in Hodes, 2013). Prenatal stress has also been tied to increased levels of DNMT1 in the adult rat hippocampus and frontal cortex, with parallel changes in hyperactivity, impaired social interactions, and decreased BDNF levels (Dong et al., 2015), though again there are few studies including effects on both sexes, particularly at early life time points which may help establish a developmental trajectory for these changes.

Along with regional and sex differences, the intensity and timing of early life stress exposure also appear to play a role in the type of methylation changes induced by prenatal stress (Mueller and Bale, 2008). One study has found that mild (2x a day, 10

minutes) and severe (3 x day, 30 minutes) stress had opposite effects on patterns of global methylation (mild stressors increased methylation, while severe stressors decreased it) in the frontal cortex of both male and female offspring on PND21 (Mychasiuk et al., 2011b). Different types of stressors (physical and psychological) have also been demonstrated to have unique effects on later behaviours (Nazeri et al., 2015). Though most stressors experienced by humans are psychological, the majority of rodent prenatal stress models have a physical component (Abe et al., 2007), highlighting the importance of developing more 'psychological' models of early life stress to examine the importance of underlying epigenetic changes in modeling vulnerability to stress and anxiety/depression.

In addition to the effects of early life stress, some long-term changes induced by the early postnatal environment also appear to be related to epigenetic influences. Increased levels of DNMT1 underlie some of the effects of maternal licking and grooming (LG) on programming the HPA axis response (Zhang et al., 2010b), potentially contributing to some of the effects of maternal separation or environmental enrichment on later behaviour (reviewed in Kofink et al., 2013). Indeed, repeated maternal separation has been linked to increased methylation of the arginine vasopressin (AVP) gene in the PVN (Murgatroyd and Spengler, 2012), while postnatal 'abuse' in a rodent model resulted in hypermethylation of BDNF in the prefrontal cortex (Roth et al., 2009), though these effects are both sex and age specific (Blaze et al., 2013). Interestingly, a clinical study also suggests that childhood maltreatment could result in increased methylation of NR3C1 (the gene for glucocorticoid receptors) in the hippocampus of suicide cases when compared to suicide cases without childhood maltreatment (Mcgowan et al., 2009).

Although little work has been done on the epigenetic impacts of early environmental enrichment, one recent study suggests that enrichment in young and older adult mice produced marked changes in hippocampal methylation patterns that correlated with improved learning and memory (Irier et al., 2014).

Taken together, these studies suggest that changes in methylation are a likely mechanism contributing to some of the effects of early life stress or enrichment on brain and behavioural changes linked to anxiety/depression - though the effects appear to be highly dependent on brain region, sex, and the type and timing of the environmental intervention. Thus, a better understanding of how environmental factors recruit the epigenetic machinery within specific brain regions to cause lasting changes in disease susceptibility and pathophysiology requires new models that incorporate sex, and more 'psychological' early interventions. Further, little research has focused on early life time points (rather than adulthood) when characterizing effects on brain and behaviour in models of stress or depression, which is an important part of understanding the developmental trajectory of these disorders. One recent study has found that rats bred for high levels of anxiety and depressive behaviour demonstrate significant decreases in the levels of DNMT1 in the basolateral and central amygdala on PND7, but these differences were absent by PND14, suggesting that some of these changes in DNMT may themselves be transient, despite their importance in the later development of anxiety and depressive behaviours (Simmons et al., 2012). Though the investigation of how early life stressors might recruit epigenetic machinery to influence neurophysiology and vulnerability to anxiety/depressive behaviours is still in its infancy, incorporating these factors (sex, psychological stressors, early life interventions) into studies of early developmental time

points could help provide new insight into the etiology and treatment of these disorders.

Previous research from our laboratory has found that a novel model of prenatal predator exposure and postnatal enhanced housing results in a number of sex-specific changes in adult anxiety and depressive behaviours. Some of these changes were predicted by behavioural changes at PND15, which suggests that there may already be some underlying neural changes at this early age that could contribute to the later development of anxiety/depressive behaviours (Green and Perrot, unpublished data, see Chapter 2). DNMT3a is particularly highly expressed in the postnatal period (Feng et al., 2005), and has been linked to both sexual differentiation (Mccarthy and Nugent, 2015; Nugent et al., 2015) and some of the effects of prenatal stress or postnatal enrichment described earlier. Thus, changes in the expression of DNMT3a at PND15 could be contributing to some of the neural, corticosterone, and behavioural changes we have previously observed following prenatal predator exposure and postnatal enhanced housing conditions, and it is likely that these changes will be dependent on brain region and sex. In order to investigate these possibilities, I examined the density of DNMT3a-ir, via immunohistochemical analysis in the PND15 brain, in several brain regions that have been associated with both anxiety/depression and epigenetic changes following stress, including the PFC, NAc-C, NAc-Sh, PVN, CeA and BLA.

3.2. Methods

3.2.1. Animals and Breeding.

Adult male and female Long-Evans hooded rats (Charles River Canada, St. Constant, Quebec) were allowed to acclimate to the colony room for at least 1 week before breeding. Vaginal smears were used to monitor females' estrous cycles, and those

in proestrus were paired overnight with a sexually experienced Long–Evans male from our colony. Vaginal smears, and the presence of sperm, the next day were used to confirm mating, and this was considered gestational day 0 (GD0). Mated females were rehoused with one other recently inseminated female until GD 12, then singly housed (with litters) for the remainder of the experiment. Rats were housed in standard colony rooms in Plexiglas cages (22 x 21 x 44 cm) with wire mesh lids, pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a black polyvinyl-carbonate (PVC) tube for enrichment, with the exception of those housed in enhanced home cages (EHC; described below). Colony rooms were maintained on a reversed 12:12 hour light:dark cycle with lights off at 07:00h, at 21±2°C. Rats had *ad libitum* access to food (Purina Lab Chow) and tap water.

Unless otherwise specified, all experimental manipulations were performed during the rats' active phase (dark cycle) under red light. Housing conditions and all experimental procedures were pre-approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and were in accordance with the guidelines stipulated by the Canadian Council on Animal Care. An effort was made to use the minimum number of animals required for statistical comparison and to minimize pain and suffering in experimental subjects.

3.2.2. Prenatal and Postnatal Environmental Manipulations

Figure 3.1 depicts the experimental timeline for perinatal manipulations, which follow the same schedule as those performed in Chapter 2. Starting on GD13, pregnant females were randomly assigned to one of four experimental conditions using prenatal naïve control (NC) or prenatal predator stress (PPS), and postnatal standard cage (SC) or

postnatal enhanced home cage (EHC), with groups as follows; 1) PPS/SC (n=5) 2) PPS/EHC (n=5) 3) NC/SC (n=4) 4) NC/EHC (n=5). From GD13-GD20, dams in the PPS groups were exposed to a predator threat 3 times a day (30 min/exposure; 08:30h, 11:30h, 14:30h). Predator threat involved transporting each dam, in a clean cage, to one of the department's 3 cat colony rooms, and, under red light, placing the cage on the floor to allow the cats to investigate/approach the cages. Dams designated as NC were similarly placed in clean cages during each exposure period, but were maintained in the colony room under red light for the duration of the 30 min exposure. On the first, third, sixth, and eighth day of the prenatal stress exposure (GD13, 15, 18, 20), fecal samples were collected for a separate experiment (described in Korgan et al., 2014). After each exposure period, PPS and NC dams were returned to their home cages and the colony room. Starting on GD20, dams were checked daily for litters. On postnatal day 0 (PND0), litters were sexed, and all pups were counted and weighed, with minimal pup handling. Those litters designated for postnatal EHC were transferred to enhanced home cages at this time and remained in EHC until sacrifice on PND15. EHC cages are approximately 2.5x the volume of standard cages and EHC cages are divided into an upper section, where food and water are available ad libitum (as well as a PVC tube for further enrichment) and a lower section simulating a burrow and containing pine shavings. Dams and pups designated as SC controls were transferred to a fresh standard cage on PND0 (See Figure 2.1). Offspring continued to be maintained on a reversed 12:12 hour light:dark cycle and had *ad libitum* access to food and water.

3.2.3. Sacrifice and Tissue Generation

On PND15, two male and two female rats from each litter were sacrificed via a

lethal dose of Euthanyl (sodium pentobarbital), injected intraperitoneally (i.p.), and brains were processed for either immunohistochemistry or western blotting (for a separate experiment).

3.2.4. Tissue Preparation and Immunohistochemistry.

Once fully anesthetized, one male and one female from each litter were perfused transcardially with 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by 4% paraformaldahyde (PFA) in PBS. Whole brains were removed from the skulls, post-fixed overnight in PFA, and then cryo-protected in 30% sucrose in 1M PBS for 2 days, before being frozen using dry-ice cooled isopentane and preserved at -80°C. Brains were sliced into serial coronal cryostat sections (12 μm) and mounted on double-subbed gelatin coated slides, allowed to dry overnight, and then stored at -80°C until processed for IHC. Tissue from 4-5 rats of each sex per treatment group was collected for analysis.

Slide-mounted tissue was first brought to room temperature, then subjected to Heat-Induced Epitope Retrieval (HIER) in 10mM sodium citrate buffer, pH 6.0, for 15 min at 95°C and allowed to cool in the buffer for 10 minutes. Slides were then rinsed in PBS for 3 x 10 min, then incubated for 45 min with agitation at room temperature in 0.1% PBS-Tween containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.03% H_2O_2 to inactivate endogenous peroxidases. After rinsing (PBS, 3 x 5 min), sections were incubated overnight at 4•C under agitation with rabbit anti-DNTM3A (sc-20703; Santa Cruz, 1:500) diluted in PBS with 1% BSA. Next, sections were rinsed (0.1% PBS-Tween, 3 x 5 min) and incubated for 90 min at room temperature with a biotinylated secondary antibody (KPL, Gaithersburg, Maryland) diluted 1/500 in PBS. Sections were rinsed (0.1% PBS-Tween, 3 x 5 min), and incubated 90 min at room

temperature in ABC complex (Vectastain, Vector Laboratories, Burlington, Ontario). After rinsing (PBS, 3 x 5 min), visualization was achieved using 0.05% diaminobenzidine diluted in PBS + 0.003% H_2O_2. Sections were then rinsed (PBS, 2 x 10 min), then dehydrated in a graded ethanol series, immersed in Histoclear (Sigma) and cover-slipped using Permount (Fisher Scientific). Both positive and negative tissue type controls were performed, as well as a no primary antibody control IHC to confirm staining effectiveness.

3.2.5. Tissue Analysis

Immunostained tissue sections were examined using an Olympus BX43 light microscope (Olympus, Markham, ON) with a 4x and 20x objective. Photomicrographs were captured on an Olympus XM10 monochrome CCD camera (Olympus, Markham, ON) controlled by cellSens imaging software with exposure time, brightness and contrast kept constant for all images. One series of tissue sections (every 6^{th} section) through several brain regions was used, with a minimum of 4 sections per brain region in each animal (n = 4-5 per group) for each antibody. The series of sections from each brain were selected with reference to an anatomical rat brain atlas (Paxinos and Watson, 2^{nd} ed.) to define the boundaries of the PFC, NAc-C, NAc-Sh, PVN, BLA, CeA (See Table 3.1 for a list of coordinates and abbreviations). The number of DNMT3a immunoreactive cells showing round/oval shape and nuclear or cytoplasmic staining in each section were manually counted in greyscale images captured thoughout the region under a 20x objective by an experimenter blind to the treatment group and sex of animals. The area (μm^2) of each region was calculated on tracings of each section using cellSens and Image J software (NIH, Bethesda, MD, USA). Densities were calculated as a function of the

number of DNMT3a-ir cells relative to the region's area, and data were expressed as the mean number of positive cells per 1000μm^2 of surface area. Sections with large tears or holes in the tissue were not counted.

3.2.6. Statistical Analysis

Differences in density of DNMT3a-ir were assessed initially in each brain region using separate 3-factor ANOVA with prenatal treatment (NC, PPS), postnatal treatment (SC, EHC), and sex (M, F) as fixed, between-subject factors. In cases of significant 3-factor interactions, 2-factor ANOVA's were performed for each sex separately and appropriate t-tests were performed upon further analysis. Tukey's HSD posthoc analysis (corrected for multiple comparisons, where appropriate) was performed. As we have previously reported sex differences in both CRF-ir and behaviour using this model (Korgan et al., 2014; 2015); Green and Perrot, unpublished data, see Chapter 2), we also performed planned a priori analyses of each sex separately. In all cases, an α level of 0.05 was considered an acceptable error level. All analyses were performed in SPSS software (v23, SPSS Inc.).

3.3. Results

3.3.1. Prefrontal Cortex

ANOVA analyses revealed a significant three-way interaction between sex, prenatal, and postnatal conditions $F(1, 29) = 23.28$, $p < 0.001$, $\eta_p^2 = 0.445$ (see Figure 3.2). In females, a significant prenatal treatment by postnatal treatment interaction was found ($p < 0.001$, $\eta_p^2 = 0.563$) and further analyses revealed that relative to NC/SC females, NC/EHC females ($p < 0.001$) and PPS/SC females ($p < 0.001$) had increased

DNMT3A-ir levels. Females exposed to both PPS and EHC had significantly decreased levels of DNMT3A-ir relative to PPS/SC females ($p = 0.003$) and NC/EHC females ($p = 0.033$). In males, there was a significant main effect of postnatal treatment, with significantly higher DNMT3A-ir in EHC compared to SC ($p = 0.001$). Females had lower levels of DNMT3A-ir relative to males in the NC/SC condition ($p = 0.003$) and PPS/EHC conditions ($p = 0.036$), and higher levels in PPS/SC ($p = 0.001$) with no significant differences in NC/EHC (See Figure 3.2).

3.3.2 Nucleus Accumbens

Nucleus Accumbens Shell (NAc-Sh)

In the NAc-Sh, there was a significant prenatal x postnatal interaction $F(1,26) = 18.16$, $p < 0.001$, $\eta_p^2 = 0.411$ and a sex x prenatal interaction $F(1,26) = 27.14$, $p = 0.004$, $\eta_p^2 = 0.511$ (See Figure 3.3). Analysis of the prenatal x sex interaction revealed that in males, but not females, PPS significantly decreased DNMT3A-ir levels ($p < 0.001$), and that females had significantly less DNMT3A-ir than males in the NC conditions ($p = 0.028$), but significantly more DNMT3A-ir than males in the PPS conditions ($p = 0.003$). The prenatal x postnatal interaction revealed that PPS decreased DNMT3A-ir levels in EHC conditions ($p = 0.008$), but not in SC. Similarly, EHC increased DNMT3A-ir in NC/EHC animals compared to NC/SC ($p = 0.034$), but decreased DNMT3A-ir in PPS/EHC compared to PPS/SC ($p = 0.049$).

When analyzed separately by sex, we see that in males, there is a significant prenatal x postnatal interaction $F(1,14) = 18.065$, $p = 0.001$, $\eta_p^2 = 0.563$ as well as the significant main effect of PPS, where prenatal stress decreased DNMT3a-ir ($p < 0.001$, $\eta_p^2 = 0.737$) relative to NC. Posthoc analyses of the interaction revealed that NC/SC males

had significantly less DNMT3a-ir density than NC/EHC males ($p = 0.019$), and a significantly higher density than PPS/EHC males ($p = 0.009$). NC/EHC males also had a significantly higher DNMT3a-ir than both PPS/SC ($p = 0.001$) and PPS/EHC ($p < 0.001$). In females, we see a trend towards a prenatal x postnatal interaction $F(1,12) = 4.63$, $p = 0.052$, $\eta_p^2 = 0.279$. Posthoc analyses revealed that this was driven by PPS/SC having a greater density of DNMT3a-ir than all other groups, NC/SC ($p = 0.014$), NC/EHC ($p = 0.049$), and PPS/EHC ($p = 0.035$). See Figure 3.3.

Nucleus Accumbens Core (NAc-C)

In the NAc-C, there was a significant prenatal x postnatal interaction $F(1,26) = 16.23$, $p < 0.001$, $\eta_p^2 = 0.384$ and a sex x prenatal interaction $F(1,26) = 10.03$, $p = 0.004$, $\eta_p^2 = 0.278$. There were also main effects of sex $F(1,26) = 4.53$, $p \leq 0.04$, with females having more DNMT3A-ir overall than males. Analysis of the prenatal x sex interaction revealed that in females, but not males, PPS significantly increased DNMT3A-ir levels ($p = 0.031$). In the NC condition, males and females were not significantly different from each other ($p = 0.542$, $\eta_p^2 = 0.027$), while following prenatal stress females had significantly higher densities of DNMT3a-ir than males ($p = 0.008$, $\eta_p^2 = 0.361$). Posthoc analyses of the prenatal x postnatal interaction revealed that PPS increased DNMT3A-ir levels in SC conditions ($p = 0.003$), but not in EHC conditions. Similarly, EHC increased DNMT3A-ir in NC animals ($p = 0.007$), but not in PPS animals.

When analyzed separately by sex, we see that in males, there is a significant prenatal x postnatal interaction $F(1,14) = 57.889$, $p < 0.001$, $\eta_p^2 = 0.805$, where NC/EHC males had significantly higher DNMT3a-ir than all other groups (all p's ≤ 0.01), and PPS/SC males had significantly higher DNMT3a-ir than both NC/SC ($p = 0.001$), and

PPS/EHC ($p = 0.002$). In females, however, only the main effect of PPS remained $F(1,12) = 6.511$, $p = 0.025$, $\eta_p^2 = 0.352$. See Figure 3.3.

3.3.3. PVN

ANOVA analyses revealed a significant main effect of prenatal stress $F(1,26) = 17.65$, $p < 0.001$, $\eta_p^2 = 0.404$), with PPS significantly increasing overall levels of DNMT3A-ir compared to NC. A trend towards a main effect of sex was also found $F(1,26) = 3.606$, $p = 0.069$, $\eta_p^2 = 0.122$, with females having less DNMT3A-ir than males.

When analyzed separately by sex, the only effect is again the main effect of prenatal condition in both males $F(1,14) = 6.552$, $p = 0.023$, $\eta_p^2 = 0.319$, and females $F(1,12) = 14.744$, $p = 0.002$, $\eta_p^2 = 0.551$, where PPS increased DNMT3a-ir density. See Figure 3.4.

3.3.4. Amygdala

Basolateral Amygdala (BLA)

ANOVA revealed significant interactions between prenatal x sex, $F(1,25) = 4.52$, $p \leq 0.044$, and postnatal x sex $F(1,25) = 7.45$, $p = 0.011$ (See Figure 3.5). There were also significant main effects of sex $F(1,25) = 10.67$, $p = 0.003$, prenatal treatment, $F(1,25) = 49.87$, $p < 0.001$, and postnatal treatment $F(1,25) = 14.93$, $p = 0.001$. Analysis of the prenatal x sex interaction revealed that in both males ($p = 0.043$) and females ($p < 0.001$), PPS significantly increased DNMT3A-ir levels. In NC conditions, but not PPS, males had significantly higher levels of DNMT3A-ir than females ($p = 0.016$). The postnatal x sex interaction revealed that in males, but not females, EHC decreased DNMT3A-ir levels ($p = 0.005$). In SC conditions, but not EHC, males had significantly higher levels of DNMT3A-ir than females ($p = 0.012$).

When analyzed separately by sex, in males, we again see a significant main effect of prenatal condition $F(1,13) = 8.2$, $p = 0.013$, $\eta_p^2 = 0.387$, where PPS significantly increased DNMT3a-ir, and a main effect of postnatal condition, $F(1,13) = 14.63$, $p = 0.002$, $\eta_p^2 = 0.529$, where EHC significantly decreased DNMT3a-ir. In females, the only effect remaining is the main effect of prenatal condition, $F(1,12) = 95.46$, $p < 0.001$), $\eta_p^2 = 0.888$, where PPS significantly increased DNMT3a-ir. See Figure 3.5.

Central Nucleus of the Amygdala (CeA)

ANOVA revealed a significant main effect of prenatal treatment $F(1,25) = 57.89$, $p \leq 0.001$, $\eta_p^2 = 0.698$, where PPS increased DNMT3A-ir density relative to NC conditions.

When analyzed separately by sex, the only effect is again the main effect of prenatal condition in both males $F(1,13) = 24.601$, $p = 0.00003$, $\eta_p^2 = 0.654$ and females $F(1,12) = 37.311$, $p = 0.00005$, $\eta_p^2 = 0.757$, where PPS increased DNMT3a-ir density. See Figure 3.6.

3.4. Discussion

The effects of prenatal predator stress (PPS) with or without a postnatal enhanced housing condition (EHC) on DNMT3a-ir densities were both sex and region specific. In males, relative to naïve control, exposure to prenatal stress increased DNMT3a-ir in the PVN, NAc-C, BLA and CeA, but not the NAc-Sh or PFC, while housing in an enhanced home cage after birth increased DNMT3a-ir in the PFC, as well as the NAc-Sh and NAc-C, and decreased DNMT3a-ir in the BLA. Providing an enhanced home cage to those that had been subjected to prenatal stress returned density levels to those of NC/SC in the NAc-C and BLA, and decreased them in the NAc-Sh. In females, prenatal stress

increased DNMT3a-ir in all brain regions studied, including the PFC, NAc-C, NAc-Sh, PVN, BLA, and CeA. Housing naïve control animals in an enhanced home cage increased levels of DNMT3a-ir density in the PFC, while housing those that were prenatally stressed in the enhanced cage returned levels to those of naïve control standard housed animals in the PFC and NAc-Sh. In the NAc-C, levels of DNMT3a-ir were decreased in prenatally stressed animals housed in enhanced home cages below those housed in standard cages, but remained higher than naïve control animals housed in standard cages. Baseline sex differences were found in DNMT3a-ir density in the PFC and BLA, while prenatal predator stress significantly increased density in females relative to males in the NAc-C and NAc-Sh, and enhanced home cage housing increased density in the male NAc-Sh compared to females (See Table 3.2 for a summary of effects).

3.4.1. Effects of Prenatal Predator Stress (PPS)

PFC

In the medial PFC (mPFC) PPS increased the density of DNMT3a-ir in female, but not male, juveniles. The mPFC is known to have connections with a number of other limbic brain regions, including the amygdala, hippocampus, and BNST, as well as GABA, 5-HT, and dopamine systems, and has been highlighted as an important contributor to the regulation of stress responding, as well as in neuropsychiatric disorders (reviewed in McLaughlin et al., 2014; McKlveen et al., 2015). Changes in DNMT3a-ir in this area may therefore be impacting some of these connections or neurotransmitter systems related to a vulnerability to neuropsychiatric disorders. Interestingly, some recent research has suggested that chronic exposure to corticosterone in adulthood can result in a persistent decrease in PFC spine density, which did not recover after a washout period,

and corresponded to a decreased sucrose preference. This suggests that CORT-induced alterations to the PFC may also contribute to anhedonia (Gourley et al., 2013). As we have previously demonstrated an increase in anhedonia in females following PPS (Green and Perrot, unpublished data, see Chapter 2), and here we find a significant female-specific increase in DNMT3a-ir, it is possible that increases in DNMT3a methylation levels in the mPFC could be contributing to our previously observed behavioural findings.

The female-specific effects of PPS in the PFC are particularly interesting in light of the data presented in Chapter 2 in which some of the significant group differences in depressive behaviours following prenatal stress did not emerge until adulthood (Green and Perrot, unpublished data; see Chapter 2). In the clinical literature, sex differences in the incidence rates of depression do not appear until later puberty, and often correlate with early life stress in women (reviewed in Hammerslag and Gulley, 2015). The PFC is one of the last cortical structures to mature, with cortical volume and connectivity continuing to develop throughout adolescence (reviewed in Wright and Perrot, 2012), and it is a key modulator of stress responding. There are significant sex differences in both the timing and type of changes to the PFC during adolescence, including rates of apoptosis and synaptic pruning, resulting in sex differences in the adult PFC volume (Hammerslag and Gulley, 2015). Within the PFC, epigenetic marker alterations have been associated with various indices of depression, including increased postmortem BDNF levels associated with antidepressant use, and decreased BDNF levels in cases of suicide (reviewed in Duclot and Kabbaj, 2015). Results presented in this chapter and Chapter 2 suggest that PPS could be disrupting aspects of ‘normal’ sex-specific

development in the PFC by altering patterns of methylation in the region by PND15. As some of our effects of PPS on behaviour did not emerge until adulthood, it may be that these changes disrupt the developmental trajectory of the mPFC and could be amplified/activated during the sensitive adolescent period, though further research is required to investigate this possibility.

Nucleus Accumbens

In our model, PPS appeared to have larger effects in females than males in the nucleus accumbens. In females, PPS alone increased DNMT3a-ir density in *both* the NAc-Sh and NAc-C, while in males the effect was only present in the PPS/SC in the NAc-C. Further, when collapsed across postnatal treatment, the effect of PPS in the NAc-C was significantly larger in females. Our results showing increased DNMT3a-ir density in the NAc-C following prenatal stress alone in males is consistent with previous research that has reported increased levels of DNMT3a in the NAc following chronic social defeat stress in males. These changes were associated with attenuated social interactions and increased forced swim test immobility, and blocking the increase in DNMT3a appeared to prevent these depressive behaviours (Laplant et al., 2010). The nucleus accumbens is known to be an important component of the mesolimbic dopamine system, involved in the processing of reward and motivation (reviewed in Cartier et al., 2015; Loke et al., 2015), and reduced activation in this region has been associated with anhedonia in both clinical and non-clinical populations in fMRI work (Wacker et al., 2009). Indeed, children with a history of early life stress have been shown to have abnormal development of the NAc during adolescence, which corresponded to higher rates of adolescent anhedonia, suggesting that early life stress could give rise to later adolescent

depression in part via disruptions to NAc development (Goff et al., 2013). Further, adolescent development of reward processing has been shown to involve epigenetic changes, as juvenile exposure to cannabinoids can alter normal H3K9 demethylation in the NAc-Sh, resulting in long term changes in reward sensitivity (Tomasiewicz et al., 2012). In rats, prenatal restraint stress has been associated with sucrose anhedonia in female, but not male offspring, following a subsequent 'second hit' of chronic mild stress in adulthood (Van den Hove et al., 2014). Prolonged postnatal maternal separation has been similarly associated with reduced social motivation and sucrose preference (reviewed in Pizzagalli, 2014). As discussed in Chapter 2, we have reported that our model of PPS decreased sucrose preference (a model of anhedonia) in adult female, but not male, rats exposed to prenatal predator stress, and postnatal EHC prevented these changes (Green and Perrot, unpublished data, Chapter 2), which appears to partially mirror the effects found here (without the rescue by EHC). Thus, it is possible that our model of PPS could be acting in the NAc to increase female vulnerability to later depressive symptoms, including anhedonia, via increased DNMT3a mediated methylation, either sex-specifically in the NAc-Sh, or through the larger increase in the NAc-C compared to males, though further research is required to investigate this link and the particular genes involved.

PVN

In the PVN, both males and females showed an increase in DNMT3a-ir following prenatal PPS, though the effect sizes of PPS were larger in females than males. These results are surprising, as we have previously reported effects of EHC, but not PPS, on CRF-ir (Korgan et al., 2015), and on fosB in response to seizure (Korgan et al., 2014) in

the PND15 PVN. Similarly, though we reported overall sex differences in plasma glucocorticoid levels in PND15 pups, PPS alone did not appear to increase circulating GCs in either sex (Green and Perrot, unpublished data; see Chapter 2). As PND15 is immediately following the normal stress-hyporesponsive period (reviewed in Wiedenmayer et al., 2005; Lupien et al., 2009; Bock and Braun, 2011), it may be that our altered levels of DNMT3a-ir represent changes to the PVN that are not yet reflected in changes to core components of the HPA axis, such as CRF levels or circulating GCs. Indeed, one recent study has demonstrated that exposure to immune activation via LPS on PND3 and PND5 resulted in significant changes in CRF mRNA levels in the PVN, but these changes only emerged at PND28 (Amath et al., 2012) – a similar timeline may be at work in our model. Alternatively, DNMT3A may be altering other genes or neurotransmitters in the PVN and not acting directly on density of CRF-ir neurons. Previous research has suggested prenatal stress can alter levels of apoptosis, cell proliferation, and synaptic connectivity in the fetal PVN, particularly in females (Charil et al., 2010; García-Cáceres et al., 2010), and thus our DNMT3a-ir changes could be linked to any of these other processes. For example, there are significant sex differences in the role of the GABA-B receptor during later fetal development, and disrupting GABA-B receptors at this point can lead to significant sex-specific effects on the HPA axis response to stress, as well as anxiety and depressive behaviours (Stratton et al., 2014). Though further research is required to elucidate the particular mechanisms and timing by which PPS is altering the PVN structure/function, our results suggest changes in methylation could be contributing to the well-documented long-term effects of prenatal stress on later function of the HPA axis.

Amygdala

In both the CeA and BLA, the main effect of PPS appeared to be to increase density of DNMT3a-ir, regardless of sex. The amygdala plays a well-documented role in both anxiety/fear responses, as well as social behaviours. Increased activation in the amygdala has been correlated with an increased risk of developing anxiety disorders, as well as current disorders, in children and adolescents (Blackford and Pine, 2012). Previous research has demonstrated that exposure to prenatal glucocorticoids alters several aspects of amygdala development, including decreased volume (via changes in dendritic branching), decreased dopamine levels (Oliveira et al., 2012) and altered rates of apoptosis (Zuloaga et al., 2011a). One recent study demonstrated that prenatal GC exposure resulted in significant changes in the mesolimbic dopaminergic system (in particular in the amygdala and NAc), and that these changes appeared to correlate with a number of social impairments and depressive behaviours (Borges et al., 2013). Similarly, results showed that low-dose prenatal ethanol exposure correlates with increased anxiety in both males and females as the result of changes to the structure of the BLA (Cullen et al., 2013). Our research suggests that epigenetic changes in DNMT3a-ir are present in both the BLA and CeA following prenatal stress exposure, and thus could underlie some of the other neural changes and anxiety behaviours reported following prenatal stress. Supporting this idea, one recent study showed that rats bred for low novelty responding - where low responders show increased anxiety and depressive behaviours, as well as enhanced stress sensitivity – showed decreased levels of DNMT1 compared to high responders in the PND7 BLA and CeA (Simmons et al., 2012).

Many studies report significant sex differences in the development of the amygdala, both in normal development and in response to early life insults (reviewed in (Cartier et al., 2015; Loke et al., 2015; Mccarthy and Nugent, 2015). Recent work by Auger and colleagues has demonstrated that the amygdala plays a key role in the development of sex differences in juvenile play behaviour, and that these sex differences are driven by differences in levels of methyl CpG binding protein 2 (MeCP2), which may prevent male-typical juvenile play behaviours by suppressing vasopressin in the female amygdala (Auger and Olesen, 2009; Edelmann and Auger, 2011; Kolodkin and Auger, 2011; Forbes-Lorman et al., 2012, reviewed in Auger and Auger, 2013). Similarly, there appear to be significant sex differences in the expression of DNMT3a protein in the developing amygdala on PND1, though these changes appear to be absent by PND10 (Kolodkin and Auger, 2011). In our study, we also find significant differences between naïve control males and females in DNMT3a-ir density in the BLA, but not CeA, suggesting that the timing of the expression of sex differences in DNMT3a-ir could be dependent on the nuclei of the amygdala, and that these changes could contribute to some of the baseline sex differences we reported in anxiety behaviours, or in juvenile play (Green and Perrot, unpublished data, See Chapter 2).

Beyond these baseline differences, the effects of GCs may also be sex-specific, as late gestational treatment with dexamethasone (DEX) decreases the density of calretinin, a calcium binding protein associated with GABAergic cells, in the amygdala of female, but not male offspring (Zuloaga et al., 2012a). As seen in the results of the last chapter, our model of PPS had significant, sex-specific effects on anxiety and social behaviours, decreasing rough and tumble play in male, but not female, peri-pubertal rats, and

increasing open field anxiety in adult males (Green and Perrot, unpublished data; see Chapter 2). Thus, it is somewhat surprising that levels of DNMT3a-ir are increased in *both* sexes following PPS at PND15, though the effect sizes were larger in females than males, with a 'large' effect in females, and a moderate effect in males. It is possible that the particular time point used in the present study is not capturing underlying sex differences in the effects of PPS on DNMT3a-ir methylation, as markers of the effects of prenatal stress may appear earlier or later, despite the prenatal nature of the disruption. The amygdala continues to develop from the late fetal period through adolescence, and some research has suggested that sex-specific changes in DNMT3a-ir methylation found in PND1 pups are no longer visible by PND10 (Kolodkin and Auger, 2011). Research suggests that some of the effects of prenatal stress on the amygdala (in particular on the developing GABAergic system) only begin to emerge at PND17, PND21 or PND28, depending on the markers studied (Kraszpulski et al., 2006; Brummelte et al., 2007; Ryan et al., 2014; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015). Alternatively, not all effects of prenatal stress reported are sex-specific, with both males and females responding to certain models of prenatal stress with altered anxiety/depressive behaviours (Bitanihirwe et al., 2010; Van den Hove et al., 2013). Therefore, it is possible the changes in DNMT3a-ir density in the amygdala reported here are common to both males and females following PPS, though perhaps to a slightly larger degree in females, while other epigenetic mechanisms (other DNMTs, HDACs, microRNAs, etc.) might be driving other sex-specific brain changes. Further, changes in overall DNMT3a-ir density do not provide us with information on the particular genes being methylated, and even in cases

where both males and females are showing increased methylation, the particular profile of genes being repressed may be different between the sexes.

Effects Summary

Across the board, the primary effect of our prenatal predator stressor was to increase the density of DNMT3a-ir cells, in both males and females, though females demonstrated these effects in some brain regions where males did not (NAc-Sh and PFC), as and had a larger increase in density compared to males in one region (NAc-C) where both sexes showed increased methylation. These results are in line with some previous research suggesting that a common result of chronic stress in adulthood is increased levels of methylation, in brain regions including the PVN and NAc (Laplant et al., 2010; Sterrenburg et al., 2011), while prenatal restraint stress has also been associated with increased DNMT1 in the frontal cortex and hippocampus(Dong et al., 2015). Thus, while intriguing, results for DNMT3a-ir in the PND15 brain do not appear to account for all effects of PPS previously reported in this model, and other epigenetic mechanisms (other DNMTs, HDACs, microRNAs, etc.) and time points should be examined, as well future studies designed to investigate the particular genes being methylated.

3.4.2. Effects of Environmental Enhanced Housing Condition (EHC) and Interactions with PPS

PFC

In both males and females, EHC increased DNMT3a-ir density in the PFC, though in females the combination of PPS/EHC prevented the effects of either alone. Previous research in adult animals has demonstrated that environmental enrichment in adults can act to prevent the effects of social defeat stress, and that its protective effects

are dependent on the vmPFC (Lehmann and Herkenham, 2011). Similarly, some recent research has shown that early life enrichment, and the natural maternal separations it induces, alters prefrontal GABA levels and glutamate receptor subunit levels in both males and females (Connors et al., 2015). Thus, it may be noteworthy that our early life EHC altered DNMT3a-ir in this region. Further, as discussed above in the section on the effects of PPS, the PFC has been linked to both stress resilience and anhedonia (Van den Hove et al., 2014). We have demonstrated that PPS results in sucrose anhedonia in adult females, and that this effect can be prevented by EHC (Green and Perrot, unpublished data; see Chapter 2), which is consistent with the effects we report for DNMT3a-ir. Interestingly, some research suggests that post-weaning environmental enrichment may also alter the response to the forced swim test via changes in the mPFC serotoninergic system (Brenes et al., 2008). In our research, however, the effects of EHC on the FST were less clear-cut, and more pronounced in the juvenile period than in adulthood. Given the continued development of the PFC throughout adolescence, it may be that the particular circuits involved in FST response were not sufficiently affected by our early life EHC, as has been suggested by other studies (Rosenfeld and Weller, 2012), or that our model may confer an underlying vulnerability that is only 'activated' in the presence of adult stressors (Rüedi-Bettschen et al., 2006; Mairesse et al., 2007; Tsoory et al., 2007). Indeed, some recent research suggests that changes in early maternal care can induce long-term epigenetic changes in the mPFC that are only expressed in adolescent and adult animals (Blaze et al., 2013), suggesting that our early life enhanced housing condition may have long term effects on methylation that are not completely captured at this time point.

Nucleus Accumbens

In the nucleus accumbens, the majority of the effects of EHC were in males. In males, EHC increased the density of DNMT3a-ir in the NAc-C, similar to PPS, but this effect was prevented by the combination of PPS/EHC, while in the NAc-Sh, EHC also increased DNMT3a-ir (though PPS did not), and the combination of PPS/EHC actually decreased methylation relative to controls. Interestingly, EHC also significantly increased DNMT3a-ir levels in males relative to females. In addition to the role of the NAc in anhedonia discussed previously, the NAc may also play an important role in the rewarding aspects of juvenile play behaviour (reviewed in Trezza et al., 2014), and has been linked to both social reward and social avoidance in human studies (Kohls et al., 2013). Previous research in our laboratory has demonstrated effects of both PPS and EHC on social behaviour, and the combination of PPS/EHC prevents some of the effects of either PPS or EHC alone on some juvenile social behaviours (Green and Perrot, unpublished data; see Chapter 2), while Morley-Fletcher and colleagues (Morley-Fletcher et al., 2003), have demonstrated a significant improvement effect of adolescent enrichment on social behaviours following prenatal stress, which appears to follow a similar pattern to some of our changes in the NAc-C. Similarly, other interventions in the early postnatal period, including maternal separation and changes in maternal care, have also been shown to alter aspects of reward processing in the NAc (Dimatelis et al., 2012; Lacagnina et al., 2014; Romano-López et al., 2015), as well as depressive behaviours (Réus et al., 2013b) and social play (Edelmann et al., 2013), and many of the changes induced by maternal care are linked to epigenetic processes (reviewed in Champagne, 2010; Mcgowan et al., 2011).

PVN

There were no significant effects of EHC in the PVN, either alone or to prevent the effects of PPS. This is somewhat surprising as we have previously demonstrated that EHC significantly decreased CRF-ir in both male and female pups on PND15 (Korgan et al., 2015). This suggests that either the effects of DNMT3a are either not directly involved in the effects of CRF-ir in the PVN, and that other epigenetic mechanisms are at play, or that, as suggested by some other studies (Kolodkin and Auger, 2011; Crudo et al., 2013), the changes induced are transitory, but alter the trajectory of the developmental pathway in a dynamic fashion that continues to progress after the initial methylation change is no longer apparent.

Amygdala

Although EHC had no independent effects in either the BLA or the CeA of females, EHC acted in the BLA to decrease DNMT3a-ir overall. As with the NAc, the BLA has been strongly implicated in social behaviour, with epigenetic changes driving a number of the sex differences reported in juvenile play behaviour (reviewed in Auger et al., 2011). Although we did not directly examine maternal behaviour in this paradigm, preliminary results from our laboratory suggest that postnatal EHC alters some aspects of maternal care (time on the nest, high arched back nursing), but not licking and grooming behaviour (Perrot and Korgan, unpublished data, personal communication); however, it is also possible that our EHC condition prevented effects of PPS on maternal care. Some recent research has suggested that changes in postnatal tactile stimulation (simulating licking and grooming) has sex-specific effects on juvenile play behaviour – that is, increased stimulation decreased juvenile play in males, without altering female

behaviours, and this seems to be related to levels of 5HT receptors in the amygdala (Edelmann et al., 2013). Therefore, it is possible that some of the sex-specific effects of EHC on DNMT3a-ir in our model are the result of changes in maternal behaviour.

The basolateral amygdala has also been linked to anxiety and depressive behaviours in a number of studies (Kraszpulski et al., 2006; Laloux et al., 2012; Mcewen et al., 2012; Oliveira et al., 2012; Ehrlich and Rainnie, 2015) including some suggesting a vulnerability to environmental disruption in the early postnatal period. Early life 'abuse' on PND8-12 alters adolescent depressive behaviours, and both juvenile and adolescent social behaviours, via an amygdala dependent mechanism (Raineki et al., 2012). Moreover, postnatal deprivation in male pups (PND1-10) results in both increased FST immobility and decreased levels of BDNF in the amygdala (Réus et al., 2013b), suggesting that the amygdala is sensitive to external influences during this time period on developing anxiety/depression. Using our model, the combination of PPS/EHC decreases male juvenile FST immobility, and prevents effects of PPS or EHC on adult open field line crosses and freezing behaviour, although it did not prevent effects of PPS on adult OFT center time, or FST climbing (Green and Perrot, unpublished data; see Chapter 2). As the combination of PPS/EHC in this study also resulted in decreased DNMT3a-ir density in the BLA, it is possible that methylation changes in this region are associated with the effects of EHC to prevent some (but not all) of the effects of PPS on anxiety behaviours in males.

Effects Summary

Unlike PPS, our postnatal EHC intervention had fewer effects in females than males, a different direction of effect depending on the region studied, and acted to

'rescue' the effects of PPS in a number of regions. In several regions, EHC alone showed effects similar to those of PPS alone (increasing the density of DNMT3a-ir). Environmental enrichment is often viewed as a positive intervention, preventing or rescuing the negative effects induced by stress, and is believed to act through a number of epigenetic mechanisms (Takuma et al., 2011), but some recent work has suggested that enrichment itself may act as a mild stressor. In male guinea pigs, adolescent enrichment increased basal circulating corticosterone levels, independent of prenatal stress effects, (Emack and Matthews, 2011), and increased anxiety behaviours in some rat studies (Connors et al., 2015). Early EE may act as a mild 'inoculation' stressor, altering aspects of stress responding and anxiety/depressive behaviours when applied alone, but providing resiliency in the face of later chronic stressors (reviewed in Crofton et al., 2015), allowing a faster return to baseline HPA function (Konkle et al., 2010) and preventing the effects of chronic stress on adult anxiety/depressive behaviours (Cymerblit-Sabba et al., 2013), which could explain some of the parallel effects of PPS and EHC on the brain in our model, as well as the opposite effects of the combination of the two interventions. Alternatively, as mentioned previously when discussing the effects of PPS, although our results suggest a similar direction for overall changes in DNMT3a-ir, the profile of which genes are being methylated may differ between the groups, and requires investigation. As some of our behavioural work (see Chapter 2) found stronger effects of EHC in juveniles than adults - in line with literature suggesting some early life interventions are insufficient to persist to adulthood (Leussis et al., 2012; Rosenfeld and Weller, 2012), or to prevent all changes following a more potent prenatal stressor (Darbra and Pallarès, 2011), an examination of further time points would also be valuable.

3.4.3. Concluding Remarks

Exposure to this novel model of prenatal predator stress and postnatal enhanced housing induced a number of changes to the density of DNMT3a-ir, suggesting alterations to the normal trajectory of epigenome development in brain regions that have been linked to both anxiety and depressive disorders. Some, but not all, of these changes in response to early life interventions were sex-specific, and our research also revealed some basal differences in DNMT3a-ir in a few of these regions at PND15 as well. Though this research points to an important role for DNMT3a in the progression linking early life stress, brain development, and vulnerability to neuropsychiatric disease, it is by no means a complete picture. Further studies should expand the current research to include a broader range of time points, including adolescent and adult animals, as well as other markers of epigenetic changes, including other DNMTs, HDACs, etc., and begin to investigate links to some of the downstream neurotransmitter systems that are being targeted by the changes in methylation observed.

3.5. Further Studies

In Chapter 4, we will delve further into the basal sex differences in DNMT3a-ir suggested in this study, by examining the role of androgens or anti-androgens in establishing the differences in regions of the PND15 brain observed here. In Chapter 5, we investigate the impact of our PPS/EHC model at this same time point (PND15) on the expression of a marker of GABAergic neurons - a neurotransmitter that has been previously linked to expression of DNMT3a-ir, as well as anxiety/depressive disorders and sexually dimorphic expression patterns. We will also then, in Chapter 6, examine the

impact of androgens and anti-androgens in establishing any sex differences in the GABAergic marker observed in Chapter 5 at this time point.

TABLE 3.1. LIMBIC BRAIN REGIONS IN WHICH DNMT3A-IMMUNOREACTIVITY DENSITY WAS QUANTIFIED. COORDINATES ARE FROM PAXINOS AND WATSON (2ND ED.).

Brain Region	Abbreviation	Bregma's coordinates
Medial Prefrontal Cortex	mPFC	3.2 – 2.2
Nucleus Accumbens, core	NAc-C	2.2 – 1.0
Nucleus Accumbens, shell	NAc-Sh	2.2 – 1.0
Paraventricular Nucleus of the Hypothalamus	PVN	-1.3 – -1.8
Central Nucleus of Amygdala	CeA	-1.88 – -3.3
Basolateral Nucleus of the Amygdala	BLA	-1.88 – -3.3

TABLE 3.2. SUMMARY OF THE EFFECTS OF PPS AND EHC ON LIMBIC BRAIN REGIONS IN WHICH DNMT3A-IMMUNOREACTIVITY DENSITY WAS QUANTIFIED.

Brain Region	PPS		EHC		Combination?	
PFC	≠ M	↑F	↑ M	↑ F	≠ M	<>F
NAC-C	↑ M	↑F	↑ M	≠ F	<> M	≠F
NAC-Sh	≠ M	↑F	↑M	≠ F	<> M	<> F
PVN	↑ M	↑F	≠ M	≠ F	≠ M	≠ F
BLA	↑M	↑F	↓ M	≠ F	≠ M	≠ F
CEA	↑ M	↑F	≠ M	≠ F	≠ M	≠ F

Summary of significant group effects of treatment on offspring DNMT3a-ir in limbic brain regions by prenatal predator stress (PPS) alone, enhanced housing condition (EHC) alone, or the combination of the treatments.. ↑ or ↓ indicates direction of effects, and <> indicates cases where PPS+EHC rescued/prevented effects of either alone, while ≠ indicates no effects of treatment. M = male, F = female. PFC = prefrontal cortex NAC-C = nucleus accumbens, core, NAC-Sh = nucleus accumbens, shell, PVN = paraventricular nucleus of the hypothalamus, BLA = basolateral amygdala, CEA = central amygdala.

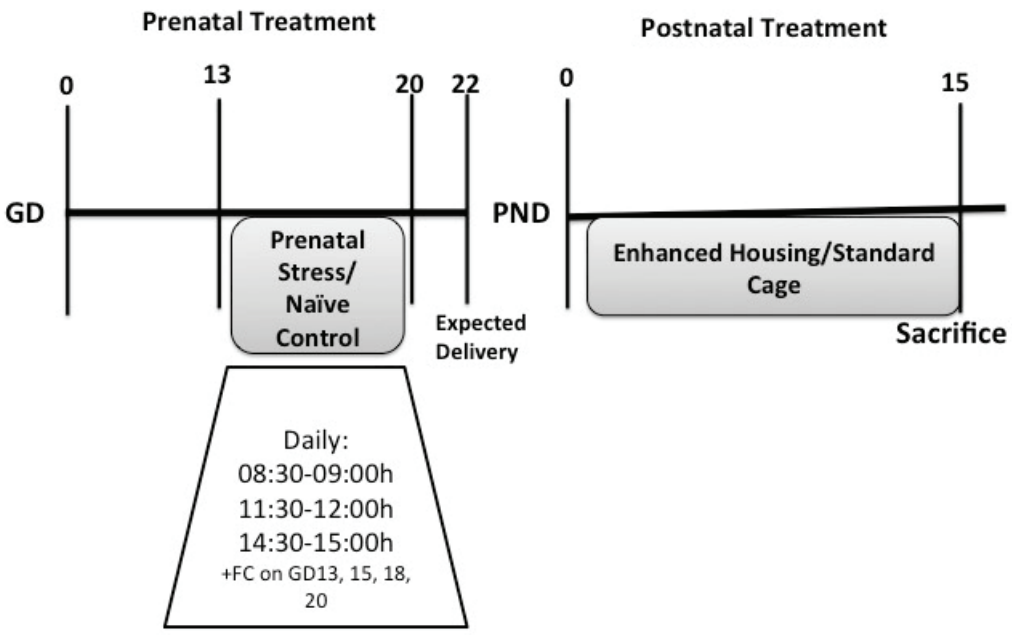

FIGURE 3.1. TIMELINE OF PRENATAL (A) AND POSTNATAL (B) TREATMENT PROCEDURES.

Dams designated to receive prenatal psychological stress (PPS) were exposed three times daily to a predator threat stressor between gestational day (GD) 13 and GD20, along with fecal collection (FC) on GD 13, 15, 18, 20. On the day of birth, postnatal day (PND) 0, dams and pups were transferred to either an Enhanced Housing Cage (EHC), or a clean Standard Cage (SC) and remained undisturbed until sacrifice on PND15.

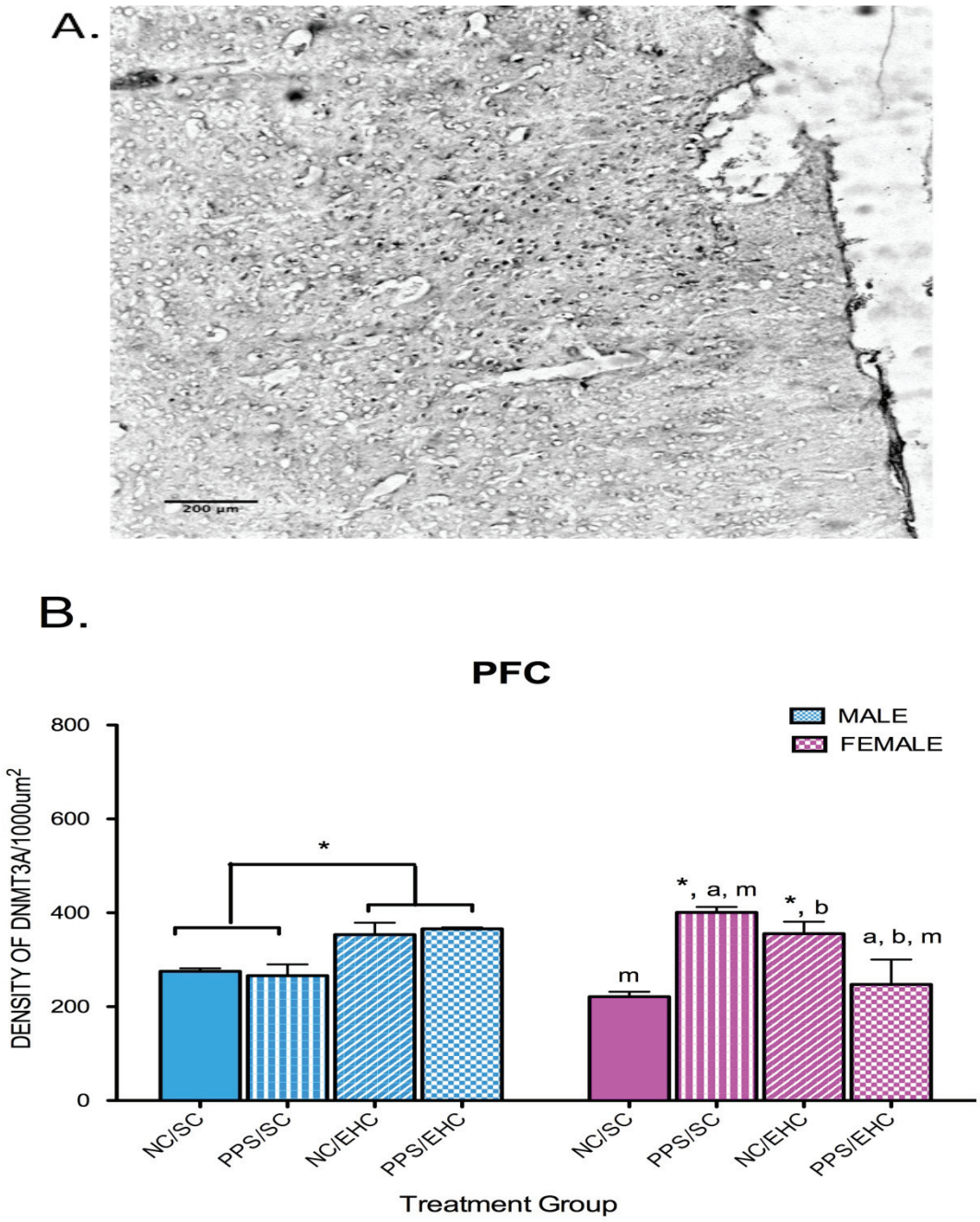

FIGURE 3.2. DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE MEDIAL PREFRONTAL CORTEX ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of DNMT3a-ir in the mPFC at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of DNMT3a-ir cells in the mPFC of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed *Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, all $p < 0.05$.

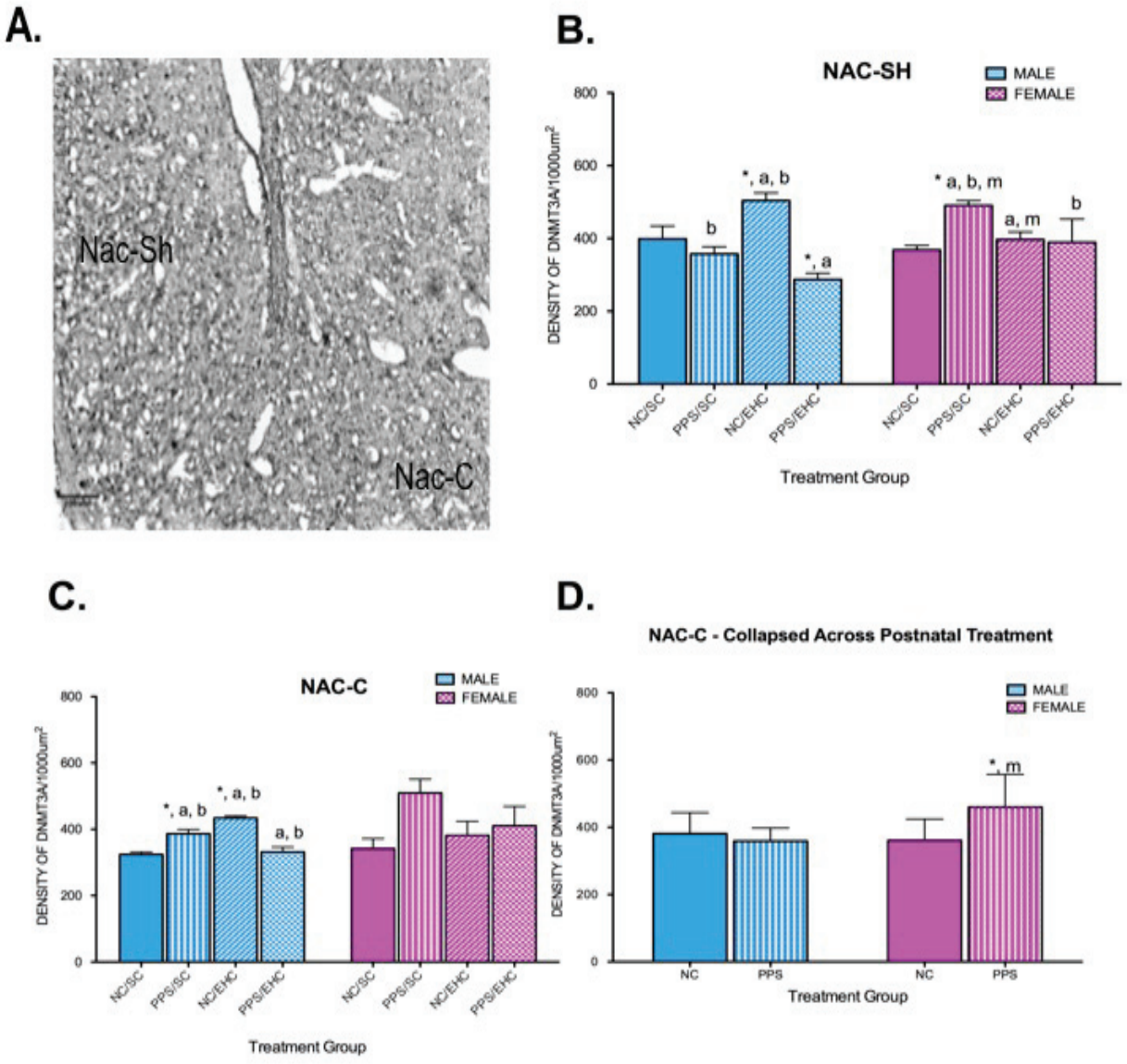

FIGURE 3.3 DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE NUCLEUS ACCUMBENS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of DNMT3a-ir in the Nucleus accumbens shell (NAc-Sh) and core (NAc-C) at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. Density of DNMT3a-ir cells in the **B.** NAc-Sh and **C.** NAc-C of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. **D.** Subjects collapsed across postnatal treatment to illustrate an effect of PPS in juvenile females Posthoc comparisons revealed * Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$

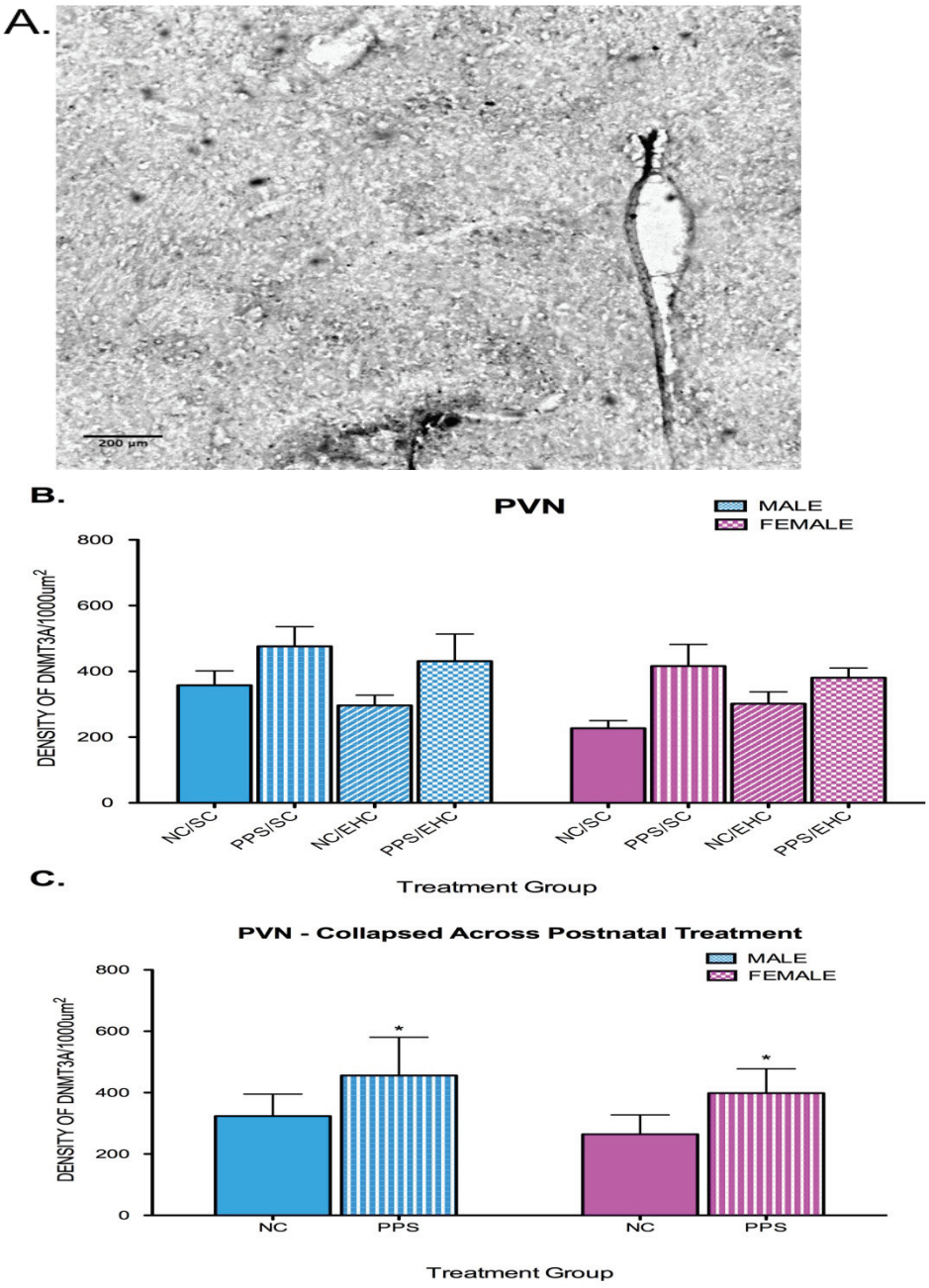

FIGURE 3.4 DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of DNMT3a-ir in the Paraventricular Nucleus of the Hypothalamus (PVN) at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of DNMT3a-ir cells in the PVN postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. **C.** Subjects collapsed across postnatal treatment to illustrate an effect of PPS in juvenile males and females. * Significantly different from same-sex NC, $p < 0.05$.

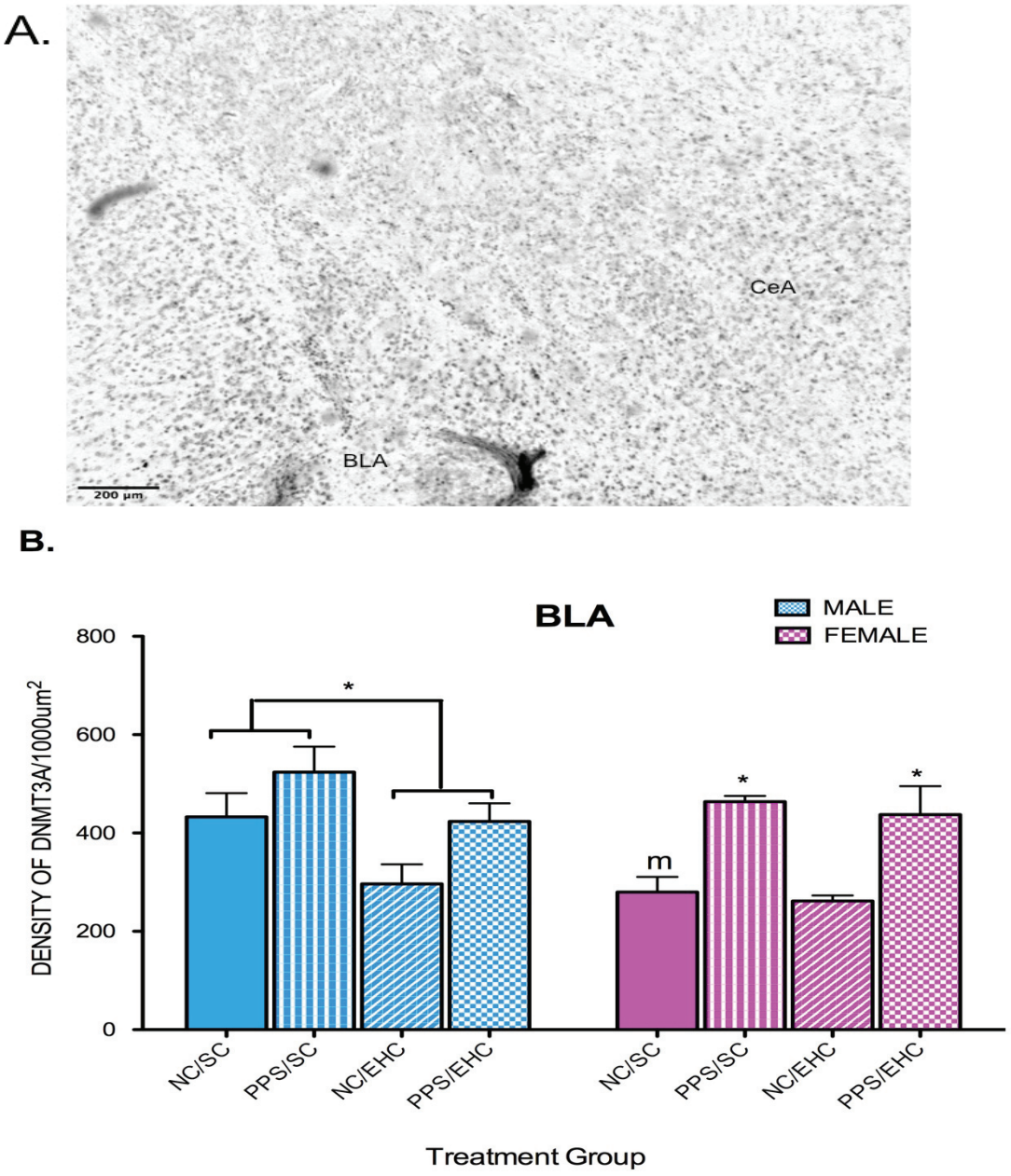

FIGURE 3.5 DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE BASOLATERAL AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of DNMT3a-ir in the Central Nucleus of the Amygdala (CeA) and Basolateral Nucleus of the Amygdala (BLA) at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. Density of DNMT3a-ir cells in the **B.** BLA on postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. * Significantly different from same-sex NC, $p < 0.05$. – significantly different from males in the same treatment

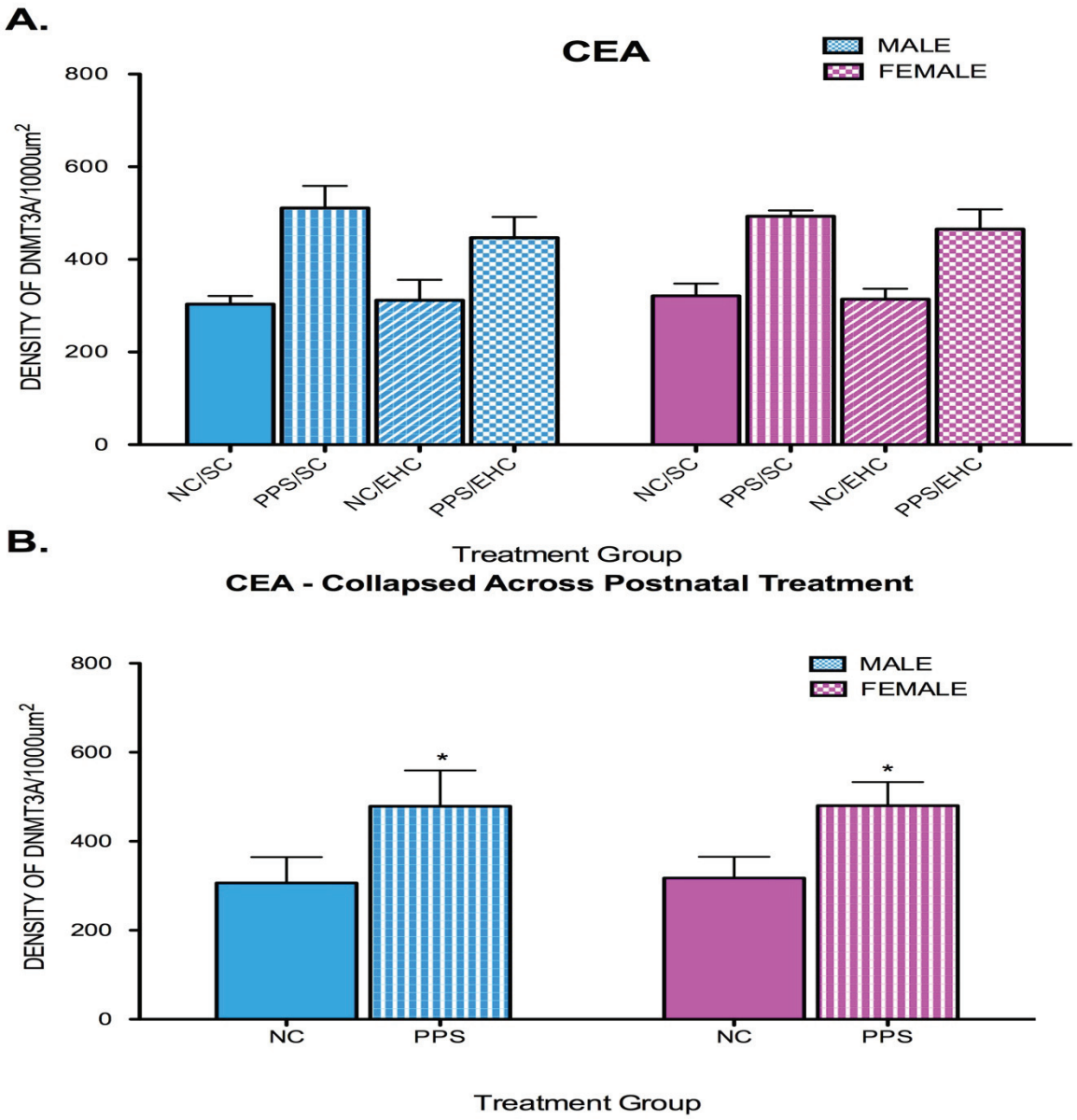

FIGURE 3.6 DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE CENTRAL AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Density of DNMT3a-ir cells in the CEA on postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. **C.** Subjects collapsed across postnatal treatment to illustrate an effect of PPS in juvenile males and females. * Significantly different from same-sex NC, $p < 0.05$.* Significantly different from same-sex NC, $p < 0.05$.

CHAPTER 4 – THE ROLE OF NEONATAL ANDROGENS IN EPIGENETIC CHANGES IN THE JUVENILE BRAIN

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4.1. Introduction

There are significant differences between the sexes in the relative risk and severity of a number of mental health disorders, including depression, with some epidemiological studies suggesting that women are twice as likely as men to develop major depressive disorders (MDD) (Silverstein, 2002; Jones, 2013; Leyfer et al., 2013). These differences are grounded in sexual dimorphism in the brain; determined by a number of genetic, hormonal, and environmental/experience factors (reviewed in Altemus et al., 2014; Bangasser and Valentino, 2014; Loke et al., 2015). Biological sex differences, which can be explored in detail in animal models, are partly influenced by hormonal differences. Throughout the lifespan, circulating gonadal hormones can have acute or transient effects on the brain and behaviour (activational effects), while, during critical periods of development, exposure to gonadal hormones can exert permanent (organizational) effects, 'wiring' the sexually dimorphic brain.

Male and female rat brains are first differentiated, in part, during a sensitive developmental period that spans across late gestation and the early postnatal period. The perinatal testes release two surges of testosterone, one on gestational day (GD) 18, and another 1-3 hours after parturition on postnatal day (PND) 0. Active metabolites of testosterone, including both androgens and estrogens, guide sexually dimorphic brain development, depending on the brain region and timing of exposure. A number of processes are impacted, such as neuronal migration, neurogenesis rates, differentiation, cell death, and synaptogenesis. Through these processes, sex differences in the size of many brain regions, as well as the connections between/within these regions are established (reviewed in Weinstock, 2011; Lenz et al., 2012; Mccarthy et al., 2012).

Though much of the existing literature has focused on the role of hormones in differentiating the brain regions and behaviours underlying reproduction (reviewed in Mccarthy et al., 2012), development of the HPA axis response to stress in males and females has been shown to be organized early in life, partly under the control of hormones (Mccormick and Mahoney, 1999; Seale et al., 2005b; Gonzalez et al., 2011; Bingham et al., 2011b; 2012). Brain regions associated with both stress responding and anxiety/depressive behaviours, including the hippocampus (Isgor and Sengelaub, 1998; Isgor et al., 2004; Zhang et al., 2010a), amygdala (Bingham and Viau, 2008; Auger et al., 2011; Jessen and Auger, 2011), BNST (Garcia-Falgueras et al., 2005; Gotsiridze et al., 2007), prefrontal cortex (Markham et al., 2013) and hypothalamus (Gonzalez et al., 2011) are affected during sensitive developmental periods.

Evidence from behavioural research supports the role of androgens in development. Some clinical studies show that prenatal exposure to endocrine disruptors such as diethylstilbestrol (DES) or bisphenol A (BPA) may increase adult risk of anxiety or depression (O'Reilly et al., 2010), a finding which mirrors several animal studies where BPA increases both anxiety and depressive behaviours (Fujimoto et al., 2006; Tian et al., 2010; Jašarević et al., 2013; Kundakovic et al., 2013). One twin study has found that environmental factors play a major role in levels of circulating testosterone in infants (Caramaschi et al., 2012), while differences in circulating levels of amniotic testosterone in boys were also linked to differences in later gray matter volume in some brain regions, including the right temporoparietal junction/posterior superior temporal sulcus, planum temporale/parietal operculum, and posterior lateral orbitofrontal cortex – areas associated with language, social, empathetic, and cognitive abilities (Lombardo et al., 2012b).

Decreased levels of prenatal (amniotic) testosterone in mid-gestation have also been associated with a lower response to reward, and an increased negative response bias in an fMRI task in pre-pubertal boys (Lombardo et al., 2012a), markers which are also associated with later depressive disorders (Lopez-Duran et al., 2012a; 2012b; Das et al., 2013; Hopkins et al., 2013; Leyfer et al., 2013). In mice, injecting females with a masculinizing dose of testosterone on the day of birth results in an 'intermediate' behavioural emotional response (between that of untreated males and females) in anxiety/depressive tasks, but only after adult exposure to chronic stress (Seney et al., 2012). Similarly, recent rat studies have found that perinatal exposure to the anti-androgen flutamide alters depressive behaviours in pre-pubertal males (Zhang et al., 2010a), and that pre-pubertal castration alters adult response to novelty in male rats (Brown et al., 2015). Organizational androgens may also play a role in the developing immune system, with perinatal flutamide treatment altering adult cytokine responses to bacterial lipopolysaccharide (LPS; Ongaro et al., 2011), and several studies have linked immune dysfunction to depression vulnerability (Anisman, 2009; Audet et al., 2014; Kim et al., 2015).

Taken together, these studies suggest that organizational hormones may contribute to the biological sex differences in both response to stress (Bangasser and Valentino, 2012; Bingham et al., 2012) and brain/behavioural changes linked to stress-related mental health disorders (Bangasser and Valentino, 2014). Sex differences in timing/trajectory of brain development in response to organizational hormones could also lead to different patterns of vulnerability to perinatal insult or stress, potentially contributing to sex differences in response to perinatal stressors – a major risk factor for

developing neuropsychiatric disorders (Laviola and Macrì, 2013; Van den Hove et al., 2014; Pereira et al., 2015). Further, prenatal stressors have themselves been shown to disrupt the usual surges of perinatal testosterone (Kapoor and Matthews, 2011; Walf and Frye, 2012; Pallarés et al., 2013b), and could contribute to the development of sex differences in some of the neural and behavioural changes observed following early life stress (Barrett et al., 2013). A better understanding of the mechanisms driving the organizational effects of these hormones in brain regions associated with anxiety and depression, could provide important clues to both sources of vulnerability to neuropsychiatric disorders, and potential therapeutic targets for intervention.

'Epigenetics' refers to modifications that alter gene expression without changing the DNA sequence, and act during cell fate determination, as well as in response to environmental influences to help 'program' long-term effects of the environment on cellular functions (Sweatt, 2009). The most widely studied epigenetic mechanisms involve regulating chromatin structure through post-translational modifications of histones, and methylation of DNA by enzymes known as DNA methyltransferases (DNMTs). DNMT enzymes act by adding methyl groups to C-5 position cytosines at CpG sites, reducing/preventing transcription of the DNA, which can then be further altered/sustained by MeCP2 (Jessen and Auger, 2011; Kigar and Auger, 2013; Mcgowan and Roth, 2015). These methylating DNMT enzymes can act either primarily to maintain pre-existing methylation patterns during cell division (i.e. DNMT1) or as *de novo* DNA methylation (i.e. DNMT3a and DNMT3b), and higher levels of these enzymes are usually considered an indication of more methylation occurring. Gene methylation often correlates with decreased gene products, but the relationship between methylation and

transcription can be more complex depending on where on the gene sequence the methyl groups are added (Mcgowan and Roth, 2015). High levels of DNMT1 are expressed in the developing brain, and are found in both postmitotic neurons, and dividing neural precursor cells. Dnmt3b appears to be key during early embryonic neurogenesis with levels decreasing before birth, while Dnmt3a is highly expressed in both postnatal and adult cells, though expression peaked after the first few postnatal weeks (Feng et al., 2005; Feng and Fan, 2009).

Epigenetic events involving histone modifications and DNA methylation are emerging as essential components of the sexual differentiation process, and appear to underlie some of organizational effects of hormones in the brain and reproductive behaviour (reviewed in Mccarthy and Nugent, 2013; 2015). In the sexually dimorphic preoptic area (POA), perinatal estradiol decreases activity of DNMTs, allowing activation of masculinizing genes, and knocking out DNMT3a can masculinize some neural markers and sexual behaviour (Nugent et al., 2015). Beyond reproduction, females rats also appear to express higher levels of DNMT3a in the amygdala on PND1; organizational hormones may trigger this difference, as treatment with either estradiol or DHT decreases levels of DNMT3a in the amygdala (Kolodkin and Auger, 2011). Similarly, perinatal exposure to the environmental endocrine disruptor bisphenol-A has been shown to increase anxiety levels, and increase activity of DNMT1 in rats (Zhou et al., 2013). BPA has also been found to dose-dependently, and sex-specifically, alter expression of DNMT1 and DNMT3a in juvenile rat cortex and hypothalamus, as well as disrupt social and anxiety behaviours, suggesting regional specificity of the effects of hormones on DNMT levels (Kundakovic et al., 2013).

While much of the research in the literature suggests that aromatization of testosterone into estradiol is a primary driver of the process for masculinizing reproductive behaviour, a growing body of evidence suggests that androgens, as well as estrogens, play important roles in masculinizing the HPA axis response and associated limbic system brain regions. Recent research in rats with a testicular feminization mutation (TfM), which results in a lack of functional androgen receptors, has demonstrated that these males have higher resting corticosterone levels, alterations of the HPA response to stress, as well as increased anxious behaviours (Zuloaga et al., 2011b; Chen et al., 2014; Hamson et al., 2014). Further, perinatal exposure to flutamide alters forced swim test depressive behaviours in pre-pubertal males (Zhang et al., 2010a). Androgens in particular are also known to be involved in adult synaptogenesis in the PFC and hippocampus, as well as cell survival rates in the hippocampus (reviewed in Celec et al., 2015) and have important modulatory effects in the adult HPA axis (reviewed in Handa and Weiser, 2014). It is possible that some of these same mechanisms could be active in early development, and contribute to neonatal patterns of brain development.

As DNMT3a is highly expressed in the late prenatal and early postnatal period (Feng et al., 2005), and has been linked to both sexual differentiation (Mccarthy and Nugent, 2015; Nugent et al., 2015) and some anxiety/depressive-linked brain and behavioural changes described above, it is a prime candidate for involvement in the hormone-driven sexual differentiation of non-reproductive brain regions, particularly those associated with stress and depression. Recent work in our laboratory has demonstrated that, at PND15, there are significant sex differences in seizure behaviour in response to prenatal stress/postnatal enhanced housing, as well as sex-specific changes in

fos-B-ir and CRF-ir in limbic brain structures (Korgan et al., 2014; 2015). Using this same model, I have demonstrated that the effects early life stress exposure in adult anxiety/depressive behaviour can be partially predicted by brain and behaviour changes in PND15 animals (Green and Perrot, unpublished data, see Chapter 2 and Chapter 3). Further, my work also suggests that there may be some basal sex differences in expression of DNMT3a-ir in some of these same brain regions (Green and Perrot, unpublished data, see Chapter 3). In order to investigate the potential role of perinatal androgens in organizing these sex differences in the brain, I have used immunohistochemical analysis to examine the expression of DNMT3a-ir in the PND15 brains of males and females after early postnatal injections of dihydrotestosterone (DHT) in females (to examine 'masculinizing' influence of androgens) or the anti-androgen flutamide (to block androgen receptor activation) in male pups. Not only is PND15 important for consistency with our past work, but it is also a time point that falls at the end of the critical period for sexual differentiation. A thorough examination of DNMT3a throughout sexual differentiation was outside the scope of this thesis and thus, instead of choosing only one time point from within the period for sexual differentiation, I chose to focus on an age at which the process is believed to have ended (reviewed in Mccarthy and Nugent, 2015; Tsai et al., 2015). In keeping with our previous work, and in light of literature suggesting potential roles of androgens in these regions described above, we have focused on the limbic associated regions including the PFC, NAc-C, NAc-Sh, PVN, CeA and BLA.

4.2. Methods

4.2.1. Animals and Breeding

Adult male and female Long-Evans hooded rats were purchased from Charles River Canada (St. Constant, Quebec). Animals were undisturbed in the colony room for at least 1 week prior to breeding. Pair-housed females were placed in a cage with a sexually experienced male for one week to allow for insemination. Mated females were removed from male cages and re-housed singly, and remained singly housed with their litters for the remainder of the experiment. Pregnant females were checked daily at the start of the dark cycle for pups beginning 20 days after being paired with a male. All rats were housed in standard colony rooms with wire mesh lids, Plexiglas cages (22 x 21 x 44 cm), pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a 15 cm black polyvinyl-carbonate (PVC) tube for enrichment. The colony rooms were maintained at 21±2°C, and on a reversed 12:12 hour light:dark cycle with lights off at 09:00h. Rats had *ad libitum* access to food (Purina Lab Chow) and tap water. All experimental procedures were pre-approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and were in accordance with the guidelines stipulated by the Canadian Council on Animal Care. An effort was made to use the minimum number of animals required for statistical comparison and to minimize pain and suffering in experimental subjects.

4.2.2. Postnatal Hormone Manipulations

Figure 4.1 depicts the experimental timeline for postnatal manipulations. On the day of birth (PND0), pups were briefly removed from the dam to be sexed and counted, and placed in a clean cage. All drugs and vehicles were administered via subcutaneous

(s.c.) injections at the nape of the neck using a 27g needle. Male pups were randomly assigned to receive injections of either flutamide or vehicle, on PND0, PND1, then every other day until PND13, with 1 pup from each litter in each group. Female pups received an injection of either vehicle or DHT on PND0 and PND1, with at least 1 pup from each litter in each group. All females then received vehicle injections every other day until PND13. Flutamide is a potent anti-androgen that inhibits androgen uptake and nuclear binding, while DHT is a potent androgen hormone (Konkle and Mccarthy, 2011). Drug doses per injection were as follows: 50µl vehicle (5% ethanol in sunflower oil), flutamide (50 mg/kg in 50ul), or DHT (100µg/50µl). These doses were chosen as they have been shown to prevent the development of typical sexual behavior and to alter the levels of CRF and GR mRNA in the adult male rat (Seale et al., 2005b), as well as alter pre-pubertal male anxiety and depressive behaviours (Zhang et al., 2010a) and to masculinize sexual behaviour, and alter the levels of CRF and GR mRNA of adult female rats (Seale et al., 2005a). At the time of drug injection on PND0, pups received a 1µl injection of India ink into the left or right hindpaw or forepaw to indicate treatment group.

4.2.3. Sacrifice and Tissue Generation

On PND15, two male and two female rats from each litter (one hormone treated and one control) were sacrificed via a lethal dose of Euthanyl (sodium pentobarbital), injected intraperitoneally (i.p.), and brains were processed for immunohistochemistry (IHC). Remaining pups were collected for a separate experiment.

4.2.4. Tissue Preparation and Immunohistochemistry

Once fully anesthetized, rats were transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by 4% paraformaldahyde (PFA) in PBS. Brains

were removed from the skulls and post-fixed overnight in PFA, then cryo-protected in 30% sucrose in 1M PBS for 2-3 days before being snap frozen using dry ice cooled isopentane, and preserved at -80°C until slicing. A cryostat was used to obtain serial coronal sections (12 μm) throughout the brain, which were mounted on double-subbed gelatin coated slides, allowed to dry overnight, and stored at -80°C until processed for IHC. Tissue from 4-5 rats per treatment group and sex was collected for analysis.

After bringing slide-mounted tissue to room temperature over 1 hour, slides were subjected to Heat-Induced Epitope Retrieval (HIER) in 10mM sodium citrate buffer, pH 6.0, for 15 min at 95°C and allowed to cool in the buffer for 10 minutes. Slides were washed in 3 x 10 minute rinses in PBS, then incubated for 45 min with agitation at room temperature in 0.1% PBS-Tween containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.03% H_2O_2 to inactivate endogenous peroxidases. Following rinses (PBS, 3 x 5 min), sections were incubated overnight with agitation at 4°C with rabbit anti-DNTM3A (sc-20703; Santa Cruz, 1:500) diluted in PBS with 1% BSA. Slides were then rinsed (0.1% PBS-Tween, 3 x 5 min) and incubated for 90 min at room temperature with biotinylated secondary antibody (KPL, Gaithersburg, Maryland) diluted 1/500 in PBS. 3 x 5 minute rinses (0.1% PBS-Tween) were completed, then slides were incubated 90 min at room temperature in ABC complex (Vectastain, Vector Laboratories, Burlington, Ontario). After rinsing (PBS, 3 x 5 min), visualization was achieved using 0.05% diaminobenzidine diluted in PBS + 0.003% H_2O_2. Sections were then rinsed (PBS, 2 x 10 min), then dehydrated in a graded ethanol series, immersed in Histoclear (Sigma) and cover-slipped using Permount (Fisher Scientific). Both positive and negative tissue type controls were performed, as well as a no primary antibody control IHC to confirm

staining effectiveness.

4.2.5. Tissue Analysis

Slides were examined using an Olympus BX43 light microscope (Olympus, Markham, ON) with a 4x and 20x objective and an Olympus XM10 monochrome digital camera (Olympus, Markham, ON) using cellSens imaging software for image capture. Exposure time, brightness and contrast were kept constant for all images. A series of tissue sections (every 6^{th} section) through several brain regions was used, with a minimum of 4 sections per brain region in each animal (n = 5-6 per group) for each antibody. The series of sections from each brain was selected with reference to an anatomical rat brain atlas (Paxinos and Watson) to define the boundaries of the PFC, NAc-C, NAc-Sh, PVN, BLA, CeA (See Table 3.1 for list of abbreviations). DNMT3a immunoreactive cells (characterized by a round/oval shape and nuclear or cytoplasmic staining) were manually counted in each section using greyscale images captured thoughout the region under a 20x objective by an experimenter blinded to the treatment group and sex of animals. Area (μm^2) of each region was calculated on tracings of each section using cellSens and Image J software (NIH, Bethesda, MD, USA). Densities were calculated as a function of the number of DNMT3a-ir cells relative to the region's area, and data were expressed as the mean number of positive cells per $1000\mu m^2$ of surface area. Sections with large tears or holes in the tissue were not counted.

4.2.6. Statistical Analyses

Differences in DNMT3a-ir were assessed initially in each brain region using a ANOVA with treatment (DHT flutamidemale emale) as the between-subject factors. In cases of significant main effects, appropriate were performed. In all cases, an α level of

0.05 was considered an acceptable error level. All analyses were performed in SPSS software (v23, SPSS Inc.).

4.3. Results

4.3.1. Prefrontal Cortex

ANOVA analyses revealed a significant interaction between sex and drug treatment $F(1, 17) = 11.63$, $p = 0.003$, $\eta_p^2 = 0.406$ (See Figure 4.2). Posthoc analyses revealed that vehicle males had significantly higher levels of DNMT3A-ir than vehicle females ($p = 0.028$). DHT treatment significantly increased DNMT3A-ir levels relative to those observed in vehicle females ($p = 0.007$), and flutamide treated males ($p = 0.026$).

4.3.2. Nucleus Accumbens

Nucleus Accumbens Shell (NAc-Sh)

ANOVA analyses revealed no significant interactions or main effects between treatment and sex, $F(1, 16) = 0.75$, $p = 0.788$, $\eta_p^2 = 0.005$. See Figure 4.3.

Nucleus Accumbens Core (NAc-C)

ANOVA analyses revealed no significant main effects or interactions between treatment and sex, $F(1, 16) = 1.206$, $p = 0.288$, $\eta_p^2 = 0.07$. See Figure 4.3.

4.3.3. PVN

ANOVA analyses revealed a significant interaction between treatment and sex, $F(1, 17) = 11.458$, $p = 0.004$, $\eta_p^2 = 0.417$ (See Figure 3.4). Posthoc analyses revealed that vehicle males had significantly higher levels of DNMT3A-ir than vehicle females ($p < 0.001$), and DHT females ($p = 0.015$), as well as a trend towards a difference with flutamide treated males ($p = 0.06$). Vehicle treated females had a significantly lower level

of DNMT3a-ir than all other treatment groups (all p's ≤ 0.015), while DHT treated females and flutamide treated males were not significantly different from each other (p = 0.503; See Figure 4.4).

4.3.4. Amygdala

Central Nucleus of the Amygdala (CEA)

ANOVA revealed a significant interaction between sex and treatment $F(1,17) = 17.928$, $p = 0.001$ $\eta_p^2 = 0.513$ (See Figure 3.5), where flutamide treated males had significantly lower levels of DNMT3A-ir than all other groups (p's ≤ 0.001). See Figure 4.5.

Basolateral Amygdala (BLA)

ANOVA revealed a significant interaction between sex and treatment $F(1,17) = 18.224$, $p = 0.001$, $\eta_p^2 = 0.517$ (See Figure 3.5) where vehicle treated males had significantly higher levels of DNMT3A-ir than all other groups (p's ≤ 0.001).

4.4. Discussion

Differences between vehicle-treated males and females in DNMT3a-ir were present in some regions examined (mPFC, BLA and PVN), but not others (NAc-C, NAc-Sh, CEA) on PND15. Blocking androgen production with flutamide treatment appeared to contribute to some (PVN and BLA), but not all (mPFC) of the sex differences, and also acted to alter DNMT3a-ir levels in the CeA, despite the lack of a sex difference in vehicle-treated animals in this region. The effects of masculinizing females with DHT treatment did not always parallel the effects of feminizing males with flutamide, resulting in intermediate DNMT phenotypes in some regions (PVN), or no effects even in areas where flutamide treatment altered DNMT3a-ir densities (BLA and CEA). The role of

androgens in DNMT3a-ir in these different regions will be discussed in detail below (see Table 4.1 for a summary).

In the mPFC, we observed a significant sex difference in levels of DNMT3a-ir in vehicle treated animals, with males having higher levels than females. In females, treatment with DHT increased density of DNMT3a-ir to levels even greater than those seen in vehicle treated males, however, treatment with flutamide resulted in intermediate levels of DNMT3a-ir that were not significantly different from either vehicle males or vehicle females. During the perinatal period, levels of androgen receptor mRNA appear to transiently increase in the PFC, from ED19-PND7, with overall levels being higher in males than females across this time frame (Mogi et al., 2015), which would facilitate a role for perinatal androgens in the development of the PFC. During typical perinatal development, there are two surges in testosterone, one peaking around GD18, and another on PND0 (reviewed in Ward et al., 2002; 2003). Thus, it is possible that both prenatal and postpartum androgens are involved in shaping the sex differences in DNMT3a-ir we see in this study, and our postpartum flutamide treatment, by blocking only postnatal androgen activity, is only disrupting one phase of androgenic activity on sexual differentiation, and resulting in the intermediate levels of DNMT3a-ir. Prenatal exposure to flutamide from GD15-21 has been shown to decrease markers of dendritic growth in the PFC of both pre-pubertal and adult male rats (Pallarés et al., 2014), supporting the role of prenatal androgens in the development of the PFC. Alternatively, both androgens and estrogens may be involved in 'normal' DNMT3a activity in the PFC, and by only blocking androgens, and not estrogens, we see this intermediate effect of flutamide on DNMT3a-ir. In either case, it may be that our super-physiological postpartum 'surge' of

DHT is sufficient to overcome either the lack of aromatized estrogens, or the absence of a prenatal testosterone surge, resulting in the high density of DNMT3a-ir seen in females given DHT.

Given the potential sex differences in the prenatal and neonatal epigenetic trajectory in the mPFC suggested here, it is possible that these epigenetic pathways are a target for some of the sex-specific effects of early life stressors on later vulnerability to anxiety/depressive disorders. The mPFC has been widely recognized as playing an important role in both the regulation of the stress response, and vulnerability to neuropsychiatric disorders (reviewed in Mcewen and Morrison, 2013), and the mPFC appears to be related to depression in clinical populations, though the precise role of the mPFC in depressive symptoms is complex (Hasler et al., 2008). Grey matter volume in the dmPFC has recently been correlated with depressive symptoms in males, but not females, in a non-clinical population, suggesting a sex specific role of the PFC in vulnerability to depression (Carlson et al., 2015). Recent results from our laboratory demonstrate that prenatal stress alters the density of DNMT3a-ir in the mPFC in a sex-specific manner (Green and Perrot, unpublished data, see Chapter 3). Therefore, disruptions to these common, hormonally driven epigenetic changes in the perinatal period could be an important pathway linking sex differences, early life stress, and the role of the mPFC in vulnerability to anxiety and depressive disorders.

It is important to note here that the ultimate direction of these effects (increasing or decreasing methylation in the brain) is difficult to conclude from our research, as DNMT3a activity may change over developmental time, with our time point reflecting a transient increase/decrease in a region. Thus, these group differences are better thought of

as demonstrating a role for androgens in recruiting epigenetic mechanisms during early development, rather than an increase or decrease in levels of methylation. Another research group has recently documented significant changes in the amount of DNMT mRNA found in the PFC across early postnatal ages, where levels of DNMT3a increased across PND4 to PND10 and PND18, but then decreased significantly by PND25, suggesting that transient changes in methylation patterns are playing a key role in the early development of this region (Westberry and Wilson, 2012). Thus, further study on underlying mechanisms, and across different time points, are required to better elucidate our observed relationships between androgens and DNMT3a in the PFC.

In the PVN, males had significantly higher levels of DNMT3a-ir than females in vehicle treated conditions, and feminizing males with flutamide, or masculinizing females with DHT, both resulted in an intermediate level of DNMT3a-ir, suggesting that postnatal androgens are responsible for some, but not all, of the sex differences observed in DNMT3a-ir in the PND15 PVN. Organizational hormones play a key role in the development of the HPA axis; exposure to testosterone, or removal of gonadal testosterone via neonatal castration, alter the magnitude of the HPA response to stress in adulthood, as well as the fos response to stress in the PVN, and these effects can be prevented by neonatal, but not adult, testosterone replacement (McCormick and Mahoney, 1999; Bingham and Viau, 2008). Similarly, treatment with either an aromatase inhibitor (1,4,6-androstatriene-3,17-dione; ATD) or flutamide, across GD13-PND20 alters both basal and stress-induced corticosterone levels in adult male rats. Both flutamide and ATD treated rats show increased levels of CRF, and decreased levels of GR, in the adult PVN, while flutamide treated rats also showed increased AVP (Seale et

al., 2005b), suggesting a role for both androgens and estrogens in the development of the PVN, and in the function of the HPA axis. Interestingly, the role of organizational hormones appears to go beyond an acute response to stress, as male pups exposed to either ATD or flutamide from PND0-PND21 also failed to show the usual decline in plasma corticosterone following repeated stress in adulthood (Bingham et al., 2011a). Our findings support this role of androgens in (partially) shaping the development of the PVN, and further, highlight the important role of epigenetic changes in the postnatal period in driving these hormonally moderated sex differences. The role of epigenetic changes in shaping sex differences in the PVN is particularly intriguing as the PVN is considered the 'final common pathway' to trigger the activation of the HPA axis. Dysregulation of the HPA axis has been implicated in the response to prenatal and adult chronic stress, as well as human anxiety and depression related disorders, all of which demonstrate significant sex differences (reviewed in Pacak et al., 1998; Xiong and Zhang, 2013; Bock et al., 2014; Handa and Weiser, 2014). Thus, epigenetic mechanisms could prove an important factor underlying interactions among sex differences in the HPA axis, early life events, and vulnerability to anxiety and depressive disorders. Indeed, our recent work has shown that sex differences are already present in plasma glucocorticoid levels at PND15 using our prenatal predator stress model (Green and Perrot, unpublished data, see Chapter 2), while other work from the laboratory has demonstrated significant sex-specific disruptions in other markers including CRF and fosB following early life interventions at this same age (Korgan et al., 2014; 2015). Thus, sex differences in DNMT3a could be playing a role in all of these effects.

In the BLA, flutamide treatment decreased DNMT3a-ir density in males to levels that were not significantly different from vehicle treated females, but surprisingly, treating females with DHT did not conversely increase levels of DNMT3a-ir.

In rodent models, the BLA continues to develop from the third week of gestation through adolescence (reviewed in Ehrlich and Rainnie, 2015), and it is possible that our chosen time point does not capture effects of DHT on the trajectory of DNMT3a-ir development that are emerging or disappearing at different time points during this period. Reflecting these dynamic changes across developmental time, research by Auger and colleagues has found that levels of DNMT3a mRNA in the amygdala are sexually dimorphic on PND1, and that a postnatal 'pulse' of DHT, similar to that used in our study, can masculinize DNMT3a mRNA levels in females, but this sex difference is no longer evident on PND10 (Kolodkin and Auger, 2011). Other studies do point to mechanistic changes occurring later in the postnatal period; with prenatal stress altering aspects of amygdala development in ways that only begin to emerge later in the second or third postnatal week (Kraszpulski et al., 2006; Brummelte et al., 2007; Ryan et al., 2014; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015). While DHT was insufficient to masculinize PND15 DNMT3a-ir in the BLA, we did observe a sex difference in vehicle treated animals. While one or two doses of testosterone within the first 24 hours after birth is well known to be sufficient to masculinize most reproductive behaviours, primarily through its aromatization to estradiol in the brain (reviewed in Schwarz and Mccarthy, 2008; McCarthy, 2009), the role of perinatal androgens and estrogens have been less thoroughly characterized in limbic brain regions, and a more complex relationship may exist. Neonatal GDX has been shown to decrease androgen receptors and vasopressin-

containing cells in the MeA, compared to sham-operated controls, and this effect can be reversed by testosterone treatment on PND1-5, suggesting that a slightly longer exposure period to both androgens and estrogens could be sufficient to alter some aspects of amygdala development (Bingham and Viau, 2008).

A recent characterization of brain steroid levels showed that while plasma levels of estradiol decrease immediately in the 48 hours after birth in both males and females, they begin to rise again on PND4 in both sexes, staying at significantly higher levels than the early postnatal period until PND10-12. This suggests a likely role for circulating plasma hormones beyond the initial postnatal gonadal surge, and into the second postnatal week (Konkle and Mccarthy, 2011). Further, results from this study demonstrated brain region-dependent sex differences in testosterone across the postnatal period, with females actually having higher testosterone than males in the cortex on PND6, as well as counterintuitive changes on PND3 following gonadectomy and adrenalectomy, where DHT levels increased in the female cortex after surgery (Konkle and Mccarthy, 2011). The authors suggest that endogenous steroidogenesis, along with regional variations in aromatase, are likely playing a previously unappreciated role in the 'typical' differentiation of the cortex and the hippocampus, both in masculinizing and defeminizing the brain, and that the prenatal and perinatal 'surge' in testosterone could trigger a cascade of endogenous changes that are not yet well understood. Recent research has confirmed that there is endogenous steroidogenesis in several other brain regions, including the hippocampus (Hojo et al., 2011). Thus, it is possible that our postnatal flutamide treatment could be acting to prevent a number of endogenous steroid actions in the amygdala, beyond disrupting postnatal gonadal steroid activity, which

could explain why flutamide appears to have more potent effects than DHT treatment in the BLA. Interestingly, in the CEA, we also found that flutamide decreased DNMT3a-ir in treated males, despite the lack of a sex difference in vehicle treated animals at this time point, which further supports the idea that flutamide could be disrupting endogenous processes in the amygdala, resulting in a phenotype different from both the 'average' male and female.

In contrast to the effects of flutamide, in both the BLA and CEA DHT did not alter DNMT3a-ir. As mentioned above, it is possible that the developmental trajectory is disrupted, but that the changes are either no longer visible, or they have yet to occur, on PND15. Alternatively, as mentioned by McCarthy and colleagues (Konkle and Mccarthy, 2011), the effects of DHT in the amygdala could require endogenous processes that need to have been 'primed' by prenatal hormone exposure – that is, without both prenatal and postnatal surges, postnatal DHT is insufficient to masculinize the PND15 amygdala, or that both androgens and estrogens are needed for the effects. Interestingly, prenatal maternal exposure to excessive androgens has been found to increase anxiety behaviour, and these changes could be prevented by either flutamide or tamoxifen (an estrogen receptor modulator), without altering adult circulating sex steroid levels, and these changes appear to correlate with a down-regulation of androgen receptors in the amygdala (Hu et al., 2015). As androgen receptors are also sexually dimorphic in their early expression, it is also possible that, without a prenatal surge, there are insufficient androgen receptors in place in the postnatal period for DHT to have the same degree of effect (Zuloaga et al., 2008).

It is also important to remember that, under typical conditions, the gonadal hormone surges do not occur in a vacuum. Recent research has begun to emphasize the importance of the sex chromosomes themselves, both in independently establishing some sex differences, and in interacting with hormonal processes (reviewed in Arnold and Chen, 2009; Ngun et al., 2011). For example, the sex-determining region of the Y chromosome (SRY) gene has recently been directly linked to dopamine synthesis and regulation is some brain regions, including midbrain dopaminergic neurons (Loke et al., 2015). Similarly, recent research has reported interactions between sex chromosome complement and the degree of aromatase activity in the BNST and anterior amygdala, where XY embryos showed higher levels of activity than XX embryos, regardless of gonadal sex. Further, the effects of estradiol or DHT on aromatase in cultured amygdala neurons appears to depend on the sex chromosomes of the cultured neurons, where DHT or estradiol increased aromatase activity in XX, but not XY embryos (Cisternas et al., 2015). This research highlights the complexity of the process of sexual differentiation in the amygdala, and provides another avenue of exploration by which our postnatal hormone treatment may have been insufficient to masculinize the CEA or BLA. Regardless of underlying mechanisms, our work adds to the growing body of literature suggesting that epigenetic mechanisms are contributing to sexual differentiation in the perinatal amygdala, which could prove key to our understanding of the role of sex differences in the amygdala both in vulnerability to anxiety/depressive disorders, and in the effects of early life stressors (Kraszpulski et al., 2006; Laloux et al., 2012; Mcewen et al., 2012; Oliveira et al., 2012; Ehrlich and Rainnie, 2015).

Surprisingly, in the NAc, we found no significant effects of either DHT or flutamide treatment on levels of DNMT3a-ir. Previous work from our laboratory indicates that the effects of prenatal predator stress on DNMT3a-ir are sex-specific at PND15 (with larger effects in females than males; Green and Perrot, unpublished data, see Chapter 3), which could indicate sex differences in the epigenetic trajectory of the NAc during perinatal development that were disrupted by our stressor, but are simply not being captured on PND15, though further research is required to investigate this possibility. Alternatively, it may be that the primary gonadal hormones driving sexual differentiation in the NAc are estrogens, not androgens, though again further research is required to investigate this possibility.

4.4.1. Concluding Remarks

The results of the present study suggest that DNMT3a is sexually dimorphic during the development of several brain regions that have been previously associated with stress responding, as well as anxiety, social, and depressive behaviours, and that androgens may be contributing to some, but not all, of the epigenetic changes in these regions. In particular, there were baseline sex differences, as well as effects of androgens, in the mPFC and PVN during this time frame, with more subtle/complex actions in the amygdala on DNMT3a-ir density. Though this research points to an intriguing role for developmental androgens, and DNMT3a, in the development of sex differences in these brain regions, it is by no means a complete picture. Further studies are needed to expand the implications of the current research to include a broader range of time points, including a detailed examination across the first 10 days of life during the period for sexual differentiation, and adolescent and adult animals. Other markers of epigenetic

changes, including other DNMTs, HDACs, etc., also need to be examined, as well as investigation of links to downstream neurotransmitter systems that are being targeted by the changes in methylation observed.

4.5. Further Studies

In Chapters 5 and 6, I will move on from the examination of epigenetic mechanisms, to investigate the role of one potential neurotransmitter system involved in behavioural and brain changes discussed in Chapters 2 and 3. In Chapter 5, I investigate the impact of the PPS/EHC model at PND15 on the expression of a marker of GABAergic neurons - a neurotransmitter that has been previously linked to expression of DNMT3a-ir, as well as anxiety/depressive disorders and sexually dimorphic expression patterns. I will then, in Chapter 6, examine the impact of androgens and anti-androgens in establishing any sex differences in the GABAergic marker observed in Chapter 5 at this time point.

TABLE 4.1. SUMMARY OF THE EFFECTS OF ANDROGENS ON LIMBIC BRAIN REGIONS IN WHICH DNMT3A-IMMUNOREACTIVITY DENSITY WAS QUANTIFIED.

Brain Region	Sex Difference?	+DHT in Females	+Flutamide in Males
PFC	M > F	↑ F	≠ M
NAC-C	M = F	≠ F	≠ M
NAC-Sh	M = F	≠ F	≠ M
PVN	M > F	↑ F	≠ M #
BLA	M > F	≠ F	↓ M
CEA	M = F	≠ F	↓ M

Summary of significant sex and treatment effects on offspring density of DNMT3a-ir by sex, or treatment with dihydrotestosterone (DHT) or the anti-androgen flutamide.. ↑ or ↓ indicates direction of effects, and ≠ indicates no effects of treatment. M = male, F = female. PFC = prefrontal cortex NAC-C = nucleus accumbens, core, NAC-Sh = nucleus accumbens, shell, PVN = paraventricular nucleus of the hypothalamus, BLA = basolateral amygdala, CEA = central amygdala.

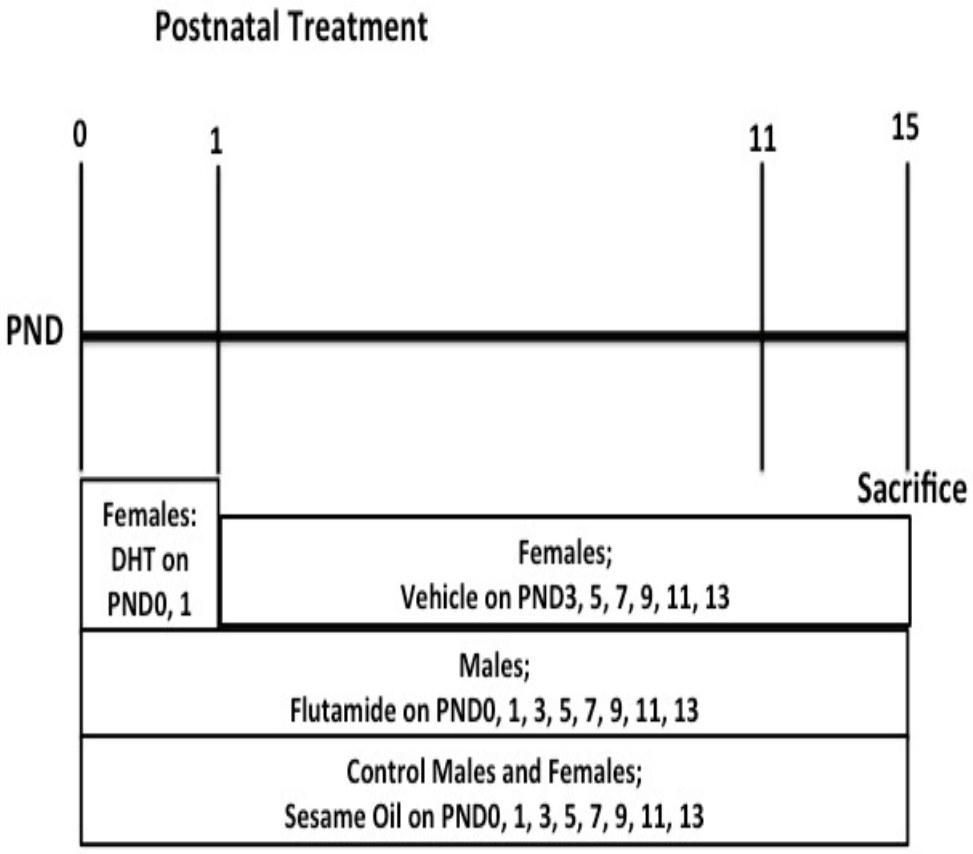

FIGURE 4.1. TIMELINE OF POSTNATAL HORMONE INJECTIONS.
Starting on the day of birth, postnatal day (PND) 0, female offspring were randomly assigned to receive subcutaneous dihyrotestosterone (DHT; 100µg/50µl) on PND0 and PN1, then vehicle control (50µl; 5% ethanol in sunflower oil) on PND3, 5, 7, 9, 11, 13, or vehicle control throughout. Male offspring were randomly assigned to receive subcutaneous flutamide (50 mg/kg in 50ul) or vehicle control on PND0, 1, 3, 5, 7, 9, 11, 13. All pups were sacrificed on PND15.

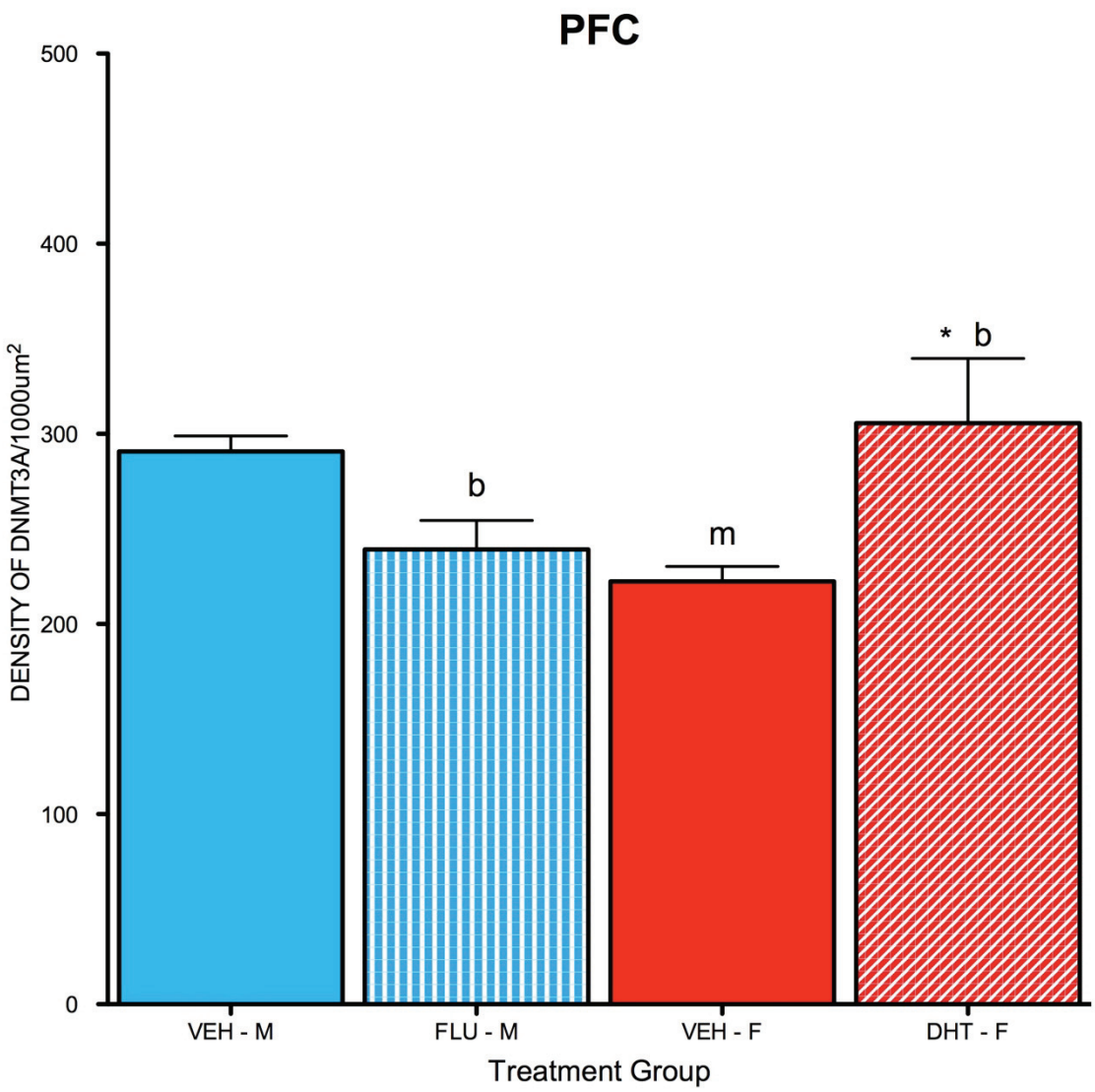

FIGURE 4.2. DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE MEDIAL PREFRONTAL CORTEX ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of DNMT3a-ir cells in the mPFC of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 4.1. Posthoc comparisons revealed * significantly different from same-sex vehice controls, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, all $p < 0.05$.

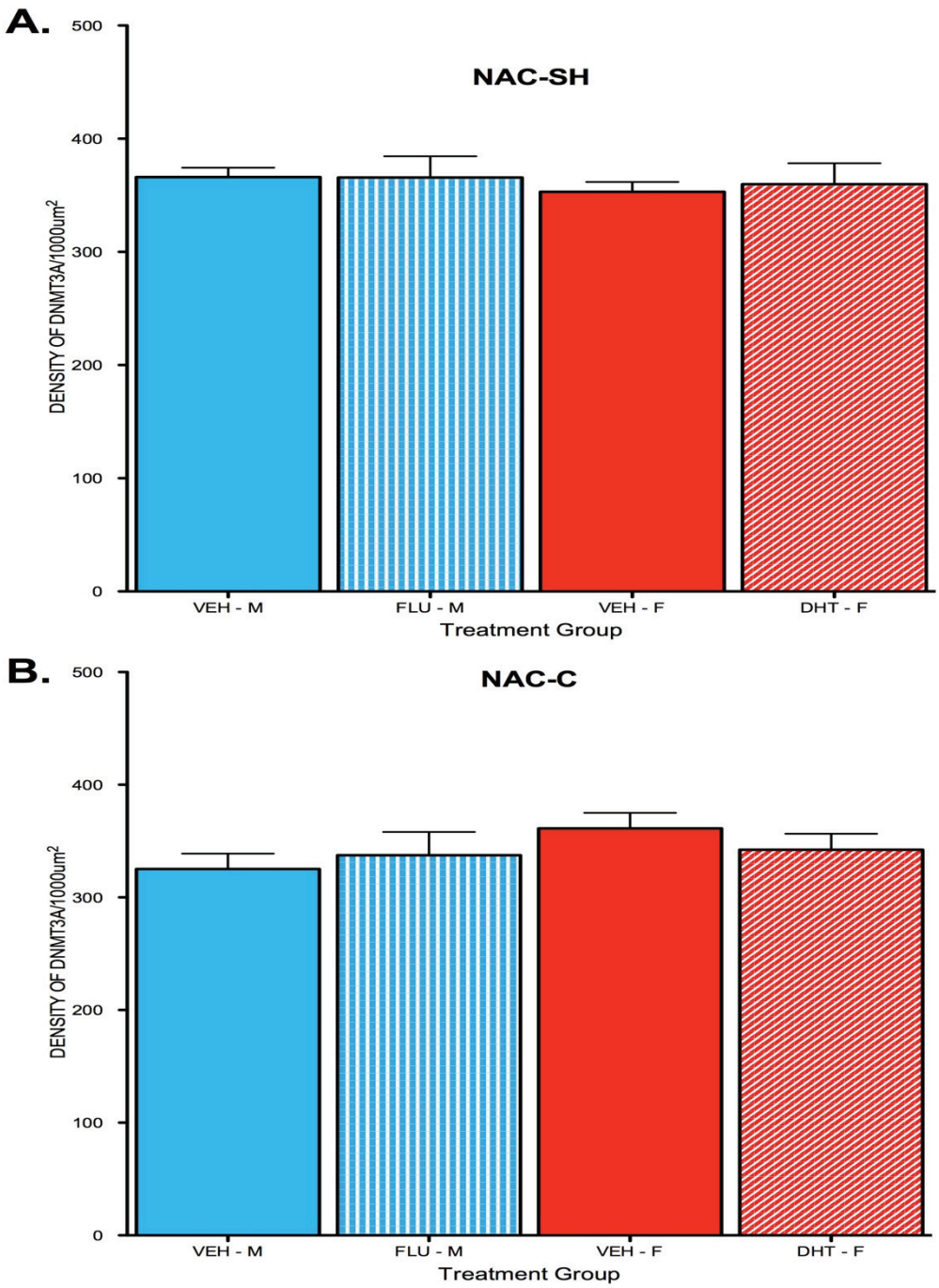

FIGURE 4.3. DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE NUCLEUS ACCUMBENS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of DNMT3a-ir cells in the **A.** Nucleus Accumbens – Shell (NAc-Sh) and **B.** Nucleus Accumbens – Core (NAc-C) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 4.1. No significant group differences were found.

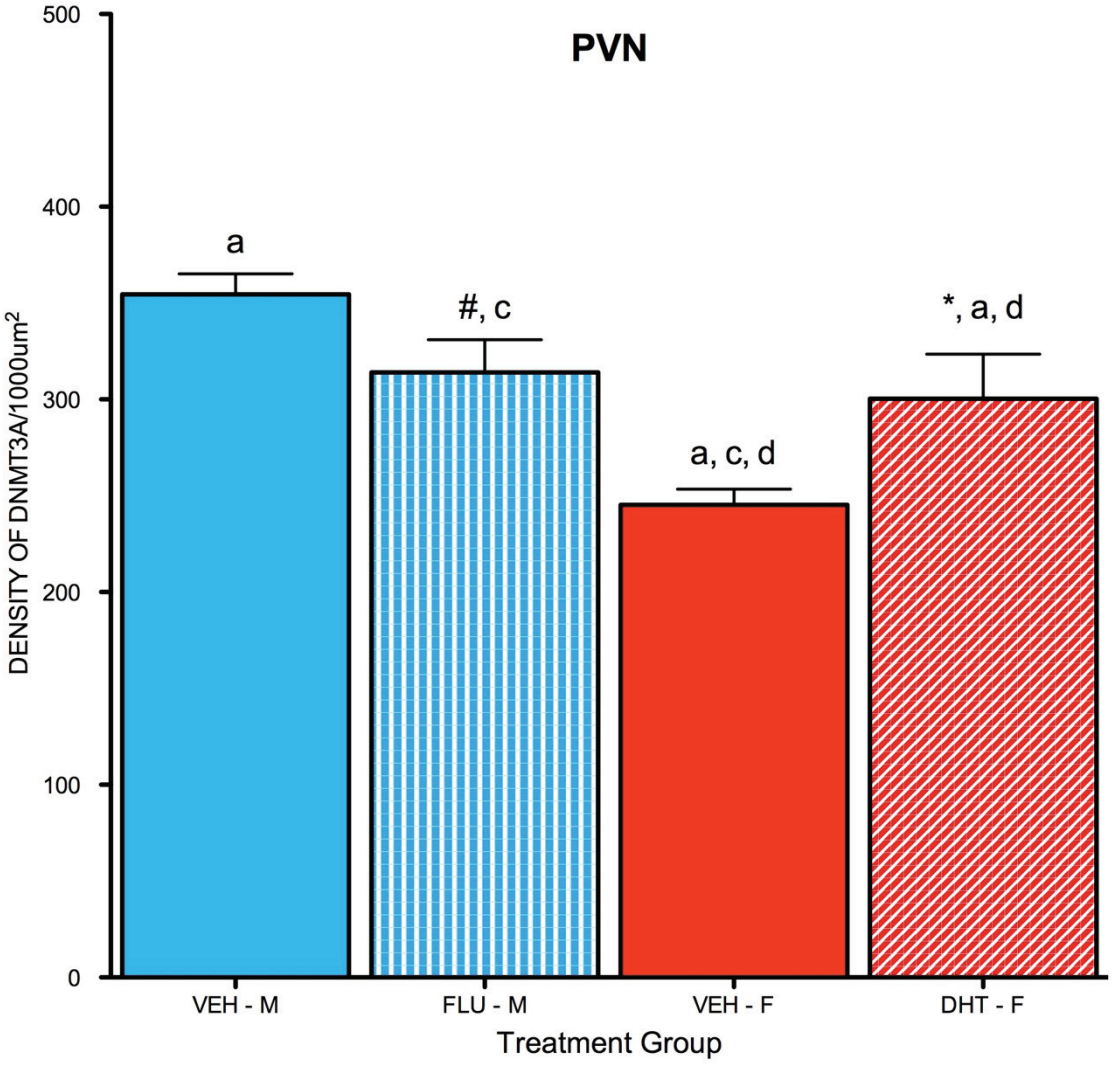

FIGURE 4.4. DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of DNMT3a-ir cells in the Paraventricular Nucleus of the Hypothalamus (PVN) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 4.1. Posthoc comparisons revealed * significantly different from same-sex vehice controls, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, all $p < 0.05$.

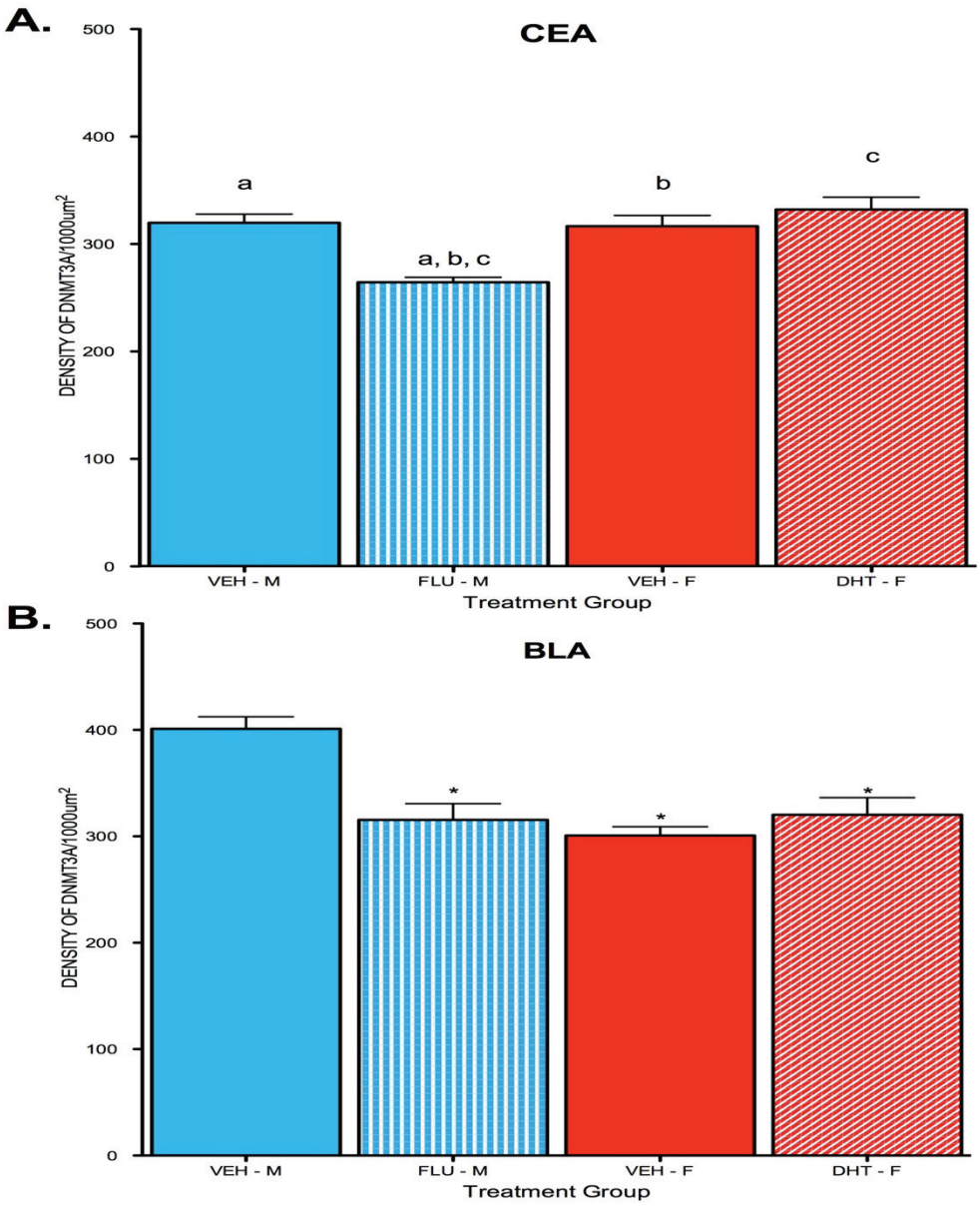

FIGURE 4.5. DENSITY OF DNMT3A-IMMUNOREACTIVE CELLS IN THE AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of DNMT3a-ir cells in the **A.** Centreal Nucleus of the Amygdala (CeA) and **B**. Basolateral Nucleus of the Amygdala (BLA) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 4.1. No significant group differences were found. Posthoc comparisons revealed * significantly different from vehice control males. Bars denoted by the same letters are significantly different from each other, all $p < 0.05$.

CHAPTER 5 – DISRUPTIONS OF JUVENILE GAD67 DENSITY FOLLOWING PRENATAL PREDATOR EXPOSURE AND EARLY LIFE HOMECAGE ENHANCEMENT

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5.1. Introduction

Disruptions to an organism's early environment can exert a profound influence on development and contribute to vulnerability to a number of neuropsychiatric disorders including anxiety, depression and schizophrenia (Hines et al., 2002; Van den Bergh et al., 2005; 2008; Davis and Sandman, 2010; Kleinhaus et al., 2013). In rats, exposing pregnant dams to prenatal stress is well established as a model of early vulnerability, altering in offspring aspects of the hypothalamic-pituitary-adrenal axis as well as contributing to the development of later anxious and depressive behaviours (Weinstock, 2008; Goel and Bale, 2009). Discovering the neural mechanisms underlying these behavioural changes is an area of active investigation, and research suggests that the type, and degree, of brain change is highly dependent on the timing of stress exposure, its duration and intensity, offspring sex, genotype and later environment, among a myriad of other factors (Darnaudéry and Maccari, 2008; Weinstock, 2008; Andersen and Teicher, 2009; Buynitsky and Mostofsky, 2009).

The type of stressor employed may play a particularly important role in modeling vulnerability to neuropsychiatric disorders (Pacak et al., 1998). In adults, restraint and social stress recruit different neural pathways and impact behaviour in unique patterns (Motta and Canteras, 2015), while prenatal restraint and a prenatal 'psychological' bystander stressor produce sex- and stressor-specific cognitive and motor disruptions (Nazeri et al., 2015), suggesting that these different types of stressors have unique effects on the brain and behaviour of offspring. Given these potential differences between restraint and other social/psychological stressors, along with human literature that

suggests that most stressors experienced in pregnancy are psychological (Abe et al., 2007), there is a clear need for more 'psychological' animal models of early life stress.

Prenatal exposure to a predator odor is an ethologically relevant, unconditioned psychological stressor, that may recruit neural circuits in a different pattern than other more physical stressors, and produces behavioural and physiological changes that persist over a long term (Blanchard et al., 1998; Morrow et al., 2000; McGregor et al., 2004; Apfelbach et al., 2005; Mashoodh et al., 2007; Muñoz-Abellán et al., 2008; Takahashi et al., 2008; Butler et al., 2011; Muñoz-Abellán et al., 2011). Prenatal predator stress might therefore provide a distinctive psychological stress paradigm to disrupt fetal and postnatal development in ways that may contribute to later anxiety/depressive vulnerability. Supporting this idea, research has suggested that prenatal predator stress has a greater impact on juvenile seizure behaviours than restraint stress (Ahmadzadeh et al., 2011) and also causes long term increases in adult anxiety behaviours and disrupts adult HPA axis function in a sex-specific manner (St-Cyr and Mcgowan, 2015).

Beyond prenatal factors, the postnatal environment is also an important time for programming later brain and behavioural responses, with postnatal handling, maternal separation (Cirulli et al., 2010) and environmental enrichment (Welberg et al., 2006), all known to interact with prenatal stress responding to change the adult brain and behaviours (Crofton et al., 2015). Environmental enrichment rearing decreases or prevents some of the effects of early life stress on adolescent and adult social, anxiety and depressive behaviours (Francis et al., 2002; Morley Fletcher et al., 2003; Welberg et al., 2006; Baldini et al., 2013). Some of these effects of enrichment may already be present in very young animals, as communal rearing (a form of early enrichment), decreases anxiety

responses in very young animals, and reduces adult depressive behaviours in the FST, and anxiety behaviours in the open field (Cirulli et al., 2010), though these effects depend on sex (D'Andrea et al., 2010). Similarly, we have demonstrated that repeated prenatal predator stress increases the likelihood and severity of seizures in the early postnatal period (PND15), and a postnatal enhanced housing condition may prevent many of post-seizure changes in the brain, though effects were larger in females than males (Korgan et al., 2014). Recently, our laboratory has used this model of prenatal predator stress to demonstrate significant, sex- and age-specific changes in anxiety and depressive behaviours, which are partially rescued by postnatal EHC (Green and Perrot, unpublished data, see Chapter 2). These results suggest that some of the underlying neural changes associated with adult anxiety/depressive behaviours may already be visible in juvenile offspring, and a better characterization of brain changes at this time point could provide insight into the impact of this model on the typical developmental trajectory, as well as providing new potential targets for interventions.

Changes to the gamma-amino–butyric-acid (GABA) system are one possible mechanism linking early life stress to later vulnerability to anxiety or depressive disorders. GABA is ubiquitous in the brain; GABAergic projections are rich in the hippocampus, amygdala, prefrontal cortex, nucleus accumbens and hypothalamus, and also help modulate the release of other neurotransmitter systems linked to depression, including dopamine, norepinephrine, and serotonin (reviewed in Croarkin et al., 2011). Further, several postmortem studies have reported region-specific decreases in GABAergic markers in depressed individuals (Rajkowska et al., 2007; Sequeira et al., 2009; Maciag et al., 2010; Guilloux et al., 2011; Gos et al., 2012; Gao et al., 2013; Seney

and Sibille, 2014) along with decreased concentrations of GABA and lower levels of inhibitory activation in several brain regions (Sanacora et al., 2004; Hasler et al., 2010; Klumpers et al., 2010; Levinson et al., 2010), though the direction of these effects is not always consistent (reviewed in Pehrson and Sanchez, 2015). Interestingly, one imaging study has reported that decreases in cortical GABA levels may still be present in patients currently in remission, suggesting that the decreased GABAergic tone could be a key developmental marker contributing to the vulnerability to depression or bipolar disorder, rather than something triggered during the disease state (Bhagwagar et al., 2008).

In animal models, the GABAergic system is itself disrupted, and chronic restraint stress in adult males has been shown to decrease the number of mPFC neurons expressing glutamate decarboxylase enzyme, 67kDa isoform (GAD67-ir; Gilabert-Juan et al., 2012). Similarly, daily adult corticosterone injections decreases GAD67 in the rodent amygdala (Lussier et al., 2013). Chronic stress in adult males has also been shown to remodel GABAergic synaptic inputs in the PVN, resulting in HPA axis hyperactivity (Miklós and Kovács, 2012). Interestingly, though isolation rearing has been shown to increase GAD67 in the rat amygdala (Gilabert-Juan et al., 2012), peri-pubertal variable stress decreased it (Tzanoulinou et al., 2014), suggesting that the type and timing of the stressor may play an important role in the direction of effects on the GABA system.

GABA may be a particularly intriguing target to examine after disruptions during sensitive periods in early life, as numbers and types of interneurons are established during this time period (reviewed in Levitt et al., 2004), and GABA modulates many important steps of brain maturation including cell proliferation, neuronal migration and synapse formation (Ben-Ari, 2002; Levitt et al., 2004; Ben-Ari et al., 2012). Further, the timing of

the maturational shift of GABAergic neurons from excitation to inhibition occurs during early postnatal life, and is both sexually dimorphic and sensitive to environmental disruptions (Galanopoulou, 2008b; He et al., 2010; Matrisciano et al., 2010; Cossart, 2011; Hyde et al., 2011; Lee et al., 2011; Ehrlich et al., 2013; Murguía-Castillo et al., 2013), which could contribute to some of the sex differences found in response to early life stress and environmental enrichment. Prenatal maternal immune activation (MIA) decreases GAD67 protein in the adult NAc, but increased levels in the mPFC (Li et al., 2015b), and prenatal dexamethasone (DEX) decreases markers of GABAergic neurons in the adult female, but not male, amygdala. Interestingly, one microarray study has found that expression of GAD may also be decreased in the frontal cortex and hippocampus of adult females, but not males, after prenatal restraint stress (Van den Hove et al., 2013), which further highlights the importance of both sex and region when interpreting the long-term effects of early life stress on GABAergic neurons.

Paralleling the literature linking prenatal stress and depression, postnatal interventions have also been found to alter the development of the GABA system. GABAergic changes in the PFC have been reported following early life environmental enrichment (Connors et al., 2015), and postnatal maternal separation decreases parvalbumin positive neurons in the PFC of pubertal rats after maternal separation (Leussis et al., 2012). Increased maternal licking and grooming, an important postpartum environmental cue, has also been shown to decrease methylation of GAD67 gene in the hippocampus, increasing GAD67 levels and altering aspects of the hippocampal GABA system (Caldji et al., 2003; Zhang et al., 2010b; reviewed in Champagne, 2013). Together, these results suggest that early environmental interventions, such as EHC, may

have important effects on the juvenile brain and behaviour that include changes to the GABAergic system, and that EHC may help rescue or prevent any effects of our prenatal predator stress on juvenile anxiety/depressive behaviours via compensatory changes in GABA.

Interestingly, changes to the GABAergic system have also been repeatedly correlated with epigenetic changes, suggesting the two processes may be working together in early development. Clinically, DNMT1 and DNMT3a have been shown to be highly expressed in GABAergic neurons in patients with neuropsychiatric disorders, suggesting a relationship between these two processes in vulnerability to anxiety/depression (Veldic et al., 2005; Ruzicka et al., 2007; Veldic et al., 2007; Poulter et al., 2008; Thompson et al., 2009; Zhubi et al., 2009; Peter and Akbarian, 2011; Kofink et al., 2013; Matrisciano et al., 2013). Similarly, in the animal literature, DNMT1 and DNMT3a expression strongly overlaps with the expression of GAD67-ir neurons (more so than glutamatergic neurons or glia) in a number of regions linked to depression, including the hippocampus, amygdala, striatum, piriform and motor cortex (Kadriu et al., 2012). More directly, overexpressing DNMT1in the brain has also been linked to decreased GAD67, along with increased anxiety behaviours, in the rodent amygdala (Zimmermann et al., 2012).

Together, the literature suggests that disruptions to the prenatal and postnatal development of GABAergic systems could be underlying some of the behavioural and HPA axis changes observed in our model of prenatal predator exposure (PPS), and postnatal enhanced housing conditions (EHC; Green and Perrot, unpublished data, Chapter 2). Some of the long-term effects on adult anxiety and depressive behaviours

were predicted by behaviours tested around PND15 (Green and Perrot, unpublished data, Chapter 2). We have also reported sex- and region-specific effects of PPS and EHC on DNMT3a-ir in several brain regions linked to mood and anxiety in juvenile animals (PND15; Green and Perrot, unpublished data, see Chapter 3), which, as discussed above, also show GABAergic changes following early life stress. In addition, GABA is often reported as a key target for epigenetic changes in some anxiety/depression models. Thus, changes in GAD67-ir that are present on PND15 could be contributing to some of the effects of PPS and EHC described in Chapter 2, and these changes are likely sex and region specific. In order to investigate these possibilities, we examined the impact of our prenatal and postnatal conditions on the density of cells expressing GAD67-ir via immunohistochemical analysis of PND15 male and female brains, investigating those regions previously established in Chapter 3, including the PFC, NAc-C, NAc-Sh, PVN, CeA and BLA.

5.2. Methods

5.2.1. Animals and Breeding.

Please note that all of the animals used in this Chapter are the same dams and offspring used in Chapter 3 to examine DNMT3a-ir (Green and Perrot, unpublished data) - immunohistochemical analyses were performed on a separate series of tissue sections through the same offspring brains. Charles River Canada (St. Constant, Quebec) provided adult male and female Long-Evans hooded rats, which were allowed to acclimate to the colony room for at least 1 week before breeding. Females' estrous cycles were monitored daily with vaginal smears and females found to be in proestrus were paired overnight with a sexually experienced Long–Evans male from our colony. Successful mating was

confirmed the next morning by the presence of sperm in vaginal smears, and was considered gestational day 0 (GD0). Mated females were then removed from males' cages and rehoused with one other recently inseminated female until GD 12. At that point, females were singly housed and remained singly housed with their litters throughout the remainder of the experiment. With the exception of those housed in enhanced home cages (EHC; described below), rats were housed in standard colony rooms in Plexiglas cages (22 x 21 x 44 cm) with wire mesh lids, pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a black polyvinyl-carbonate (PVC) tube for enrichment. The colony rooms were maintained at 21±2°C, and on a reversed 12:12 hour light:dark cycle with lights off at 07:00h. Rats had *ad libitum* access to food (Purina Lab Chow) and tap water.

Unless otherwise specified, all experimental manipulations were performed during the rats' active phase (dark cycle) under red light. Housing conditions and all experimental procedures were pre-approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and were in accordance with the guidelines stipulated by the Canadian Council on Animal Care. An effort was made to use the minimum number of animals required for statistical comparison and to minimize pain and suffering in experimental subjects.

5.2.2. Prenatal and Postnatal Environmental Manipulations

Figure 5.1 depicts the experimental timeline for perinatal manipulations. Starting on GD13, pregnant females were randomly assigned to one of four experimental conditions using prenatal naïve control (NC) or prenatal predator stress (PPS), and postnatal standard cage (SC) or postnatal enhanced home cage (EHC), with groups as

follows; 1) PPS/SC (n=5) 2) PPS/EHC (n=5) 3) NC/SC (n=4) 4) NC/EHC (n=5). From GD13-GD20, dams in the PPS groups were exposed 3 times a day (30 min/exposure; 08:30h, 11:30h, 14:30h) to a predator threat. This threat was administered by placing each dam in a clean cage and transporting her to one of the 3 cat colony rooms in our department, where the cage was placed on the floor to allow the cats to investigate. All exposures took place under red light. During each exposure period, dams designated as NC were similarly placed in clean cages but were maintained in the colony room under red light for the duration of the 30 min. On the first, third, sixth, and eighth day of the prenatal stressing (GD13, 15, 18, 20), fecal samples were collected for a separate experiment, described elsewhere (Korgan et al., 2014). After each exposure period, PPS and NC dams were returned to their home cages and the colony room. All dams were checked daily for pups starting on GD20 at the start of the dark cycle. On the day of delivery, designated postnatal day 0 (PND0), litters were sexed, and all pups were counted and weighed. This was performed quickly to minimize pup handling. Dams and pups designated to receive postnatal EHC were transferred to enhanced home cages at this time and remained in EHC until sacrifice on PND15. EHC cages are approximately 2.5x the volume of standard cages and EHC cages are divided into an upper section, where food and water are available ad libitum (as well as a PVC tube for further enrichment) and a lower section simulating a burrow and containing pine shavings. Dams and pups designated as SC controls were transferred to a fresh standard cage (described in section 2.1) on PND0. Offspring continued to be maintained on a reversed 12:12 hour light:dark cycle and had *ad libitum* access to food and water.

5.2.3. Sacrifice and Tissue Generation

On PND15, two male and two female rats from each litter were sacrificed via a lethal dose of Euthanyl (sodium pentobarbital), injected intraperitoneally (i.p.), and brains were processed for either immunohistochemistry or western blotting (for a separate experiment).

5.2.4. Tissue Preparation and Immunohistochemistry

Once fully anesthetized, one male and one female from each litter were perfused transcardially with 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by 4% paraformaldahyde (PFA) in PBS. Whole brains were removed from the skulls, post-fixed overnight in PFA, and then cryo-protected in 30% sucrose in 1M PBS for 2 days, before being frozen using dry-ice cooled isopentane and preserved at -80°C. Brains were sliced into serial coronal cryostat sections (12 μm) and mounted on double-subbed gelatin coated slides, allowed to dry overnight, and then stored at -80°C until processed for IHC. Tissues from 4-5 rats per treatment group and sex were collected for analysis.

Slide-mounted tissue was first brought to room temperature, then subjected to Heat-Induced Epitope Retrieval (HIER) in 10mM sodium citrate buffer, pH 6.0, for 15 min at 95°C and allowed to cool in the buffer for 10 minutes. Slides were then rinsed in PBS for 3 x 10 min, then incubated for 45 min with agitation at room temperature in 0.1% PBS-Tween containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.03% H_2O_2 to inactivate endogenous peroxidases. After rinsing (PBS, 3 x 5 min), sections were incubated overnight at 4•C under agitation with either mouse anti-GAD67 (MAB5406, EMD Millipore; 1:1000) diluted in PBS with 1% BSA. Next, sections were rinsed (0.1% PBS-Tween, 3 x 5 min) and incubated for 90 min at room temperature with

a biotinylated secondary antibody (KPL, Gaithersburg, Maryland) diluted 1/500 in PBS. Sections were rinsed (0.1% PBS-Tween, 3 x 5 min), then incubated 90 min at room temperature in ABC complex (Vectastain, Vector Laboratories, Burlington, Ontario). After rinsing (PBS, 3 x 5 min), visualization was achieved using 0.05% diaminobenzidine diluted in PBS + 0.003% H_2O_2. Sections were then rinsed (PBS, 2 x 10 min), then dehydrated in a graded ethanol series, immersed in Histoclear (Sigma) and cover-slipped using Permount (Fisher Scientific). Both positive and negative tissue type controls were performed, as well as a no primary antibody control IHC to confirm staining effectiveness.

5.2.5. Tissue Analysis

Processed tissue sections were examined using an Olympus BX43 light microscope (Olympus, Markham, ON) with a 4x and 10x objective and an Olympus XM10 monochrome CCD digital camera (Olympus, Markham, ON), controlled by cellSens imaging software to capture the images. Exposure time, brightness and contrast were kept constant for all images. One series of tissue sections (every 6^{th} section) through several brain regions was used, with a minimum of 4 sections per brain region in each animal (n = 4-5 per group) for GAD67-ir. The series of sections from each brain were selected with reference to an anatomical rat brain atlas (Paxinos and Watson) to define the boundaries of the PFC, NAc-C, NAc-Sh, PVN, BLA, CEA (See Table 3.1 for list of abbreviations). All GAD67-ir neurons (defined as showing a round/oval shape and primarily cytoplasmic staining patterns) present on each section were manually quantified in bilateral sections captured in greyscale images captured under a 10x objective by an experimenter blind to the treatment group and sex of the animals. (μm^2) of each region

was calculated on tracings of each section using cellSens and Image J software (NIH, Bethesda, MD, USA). Densities were calculated as a function of the number of GAD67-ir cells relative to the region's area, and data were expressed as the mean number of positive cells per 1000μm^2 of surface area. Sections with large tears or holes in the tissue were not counted.

5.2.6. Statistical Analysis

Differences in GAD67-ir density were assessed initially in each brain region using separate 3-factor ANOVA with prenatal treatment (NC, PPS), postnatal treatment (SC, EHC), and sex (M, F) as fixed, between-subject factors. In cases of significant 3-factor interactions, 2-factor ANOVA's were performed for each sex separately and appropriate t-tests were performed upon further analysis. Tukey's HSD posthoc analysis (corrected for multiple comparisons, where appropriate) was performed. As we have previously reported sex differences in both CRH-ir and behaviour using this model (Korgan et al., 2014; 2015), we also performed planned a priori analyses of each sex separately. In all cases, an α level of 0.05 was considered an acceptable error level. All analyses were performed in SPSS software (v23, SPSS Inc.).

5.3. Results

5.3.1. Prefrontal Cortex

MANOVA analyses revealed a significant interaction between prenatal x postnatal treatments, $F(1, 28) = 12.518$, $p = 0.001$, $\eta_p^2 = 0.309$ as well as an interaction between postnatal treatment x sex, $F(1, 28) = 9.1$, $p = 0.005$, $\eta_p^2 = 0.245$ (See Figure 5.2). Interestingly, the postnatal x sex interaction revealed that in males, EHC appeared to have

no effect on GAD67-ir density overall (p = 0.4), while EHC decreased GAD67-ir density in females (p = 0.005), with females having a lower density of GAD67-ir following EHC than males (p = 0.001). When analyzed separately by sex, in males, the prenatal x postnatal interaction remains (p = 0.004), where GAD67-ir is significantly reduced in PPS/SC animals compared to both NC/SC (p = 0.013) and PPS/EHC (p = 0.008), while in females only a main effect of postnatal condition remains (p = 0.004). See Figure 5.2.

5.3.2. Nucleus Accumbens

Nucleus Accumbens Shell (NAc-Sh).

In the NAc-Sh, we observed a significant interaction between prenatal treatment and sex, $F(1,28) = 4.48$, $p = 0.043$, , $\eta_p^2 = 0.138$, but nothing significant could be resolved with further examination. See Figure 5.3.

Nucleus Accumbens Core (NAc-C)

In the NAc-C, main effects of sex revealed that females had significantly lower density of GAD67-ir than males, $F(1,28) = 7.38$, $p = 0.011$, $\eta_p^2 = 0.209$, along with a main effects of postnatal treatment $F(1,28) = 5.33$, $p = 0.160$, where EHC significantly decreased GAD67-ir compared to SC (See Figure 5.4). When analyzed separately by sex, no effects of treatment were present in males, while in females, a trend towards a main effect of EHC remained $F(1,16) = 4.55$, $p = 0.052$, $p = 0.259$.

5.3.3. PVN

ANOVA analyses revealed a significant prenatal x postnatal x sex interaction, $F(1,26) = 11.63$, $p = 0.002$, $\eta_p^2 = 0.309$ (See Figure 5.5). When males were analyzed separately, there was a main effect of postnatal treatment ($p = 0.009$, $\eta_p^2 = 0.399$), and a trend towards a main effect of prenatal treatment ($p = 0.051$, $\eta_p^2 = 0.246$), where both PPS

and EHC increased GAD67-ir. Further analyses revealed that these effects were driven by PPS/EHC being significantly higher than all other groups (all p's ≤ 0.02). In females, a significant prenatal treatment by postnatal treatment interaction was found (p=0.008, η_p^2 =0.452). Further posthoc analyses revealed that, compared to NC/SC, PPS/SC had significantly higher levels of GAD67 (p = 0.025), and PPS/EHC had significantly lower levels of GAD67-ir (p = 0.007). Comparisons between the sexes revealed that females had higher levels of GAD67-ir relative to males in animals exposed to PPS/SC (p=0.01) and decreased levels compared to males in PPS/EHC (p=0.028). See Figure 5.5.

5.3.4. Amygdala

Central Nucleus of the Amygdala (CeA)

ANOVA revealed a significant prenatal x postnatal x sex interaction in the CeA, F(1,27) = 20.03, p = 0.001, η_p^2 =0.426 (See Figure 5.6). In males a significant prenatal x postnatal interaction was noted (p=0.005, η_p^2 =0.423) and further analyses revealed that NC/SC offspring had significantly higher GAD67-ir levels than both NC/EHC (p = 0.008), and PPS/SC (p = 0.005), while PPS/EHC was not significantly different from any other group. A significant interaction between pre- and post-treatments was also found for females (p=0.011, η_p^2 =0.432) and further analyses revealed that, contrary to males, PPS/SC increased GAD67-ir relative to NC/SC (p = 0.005), and PPS/EHC (p = 0.022). Comparisons between the sexes revealed that, in NC/SC offspring, females had significantly less GAD67-ir than males (p = 0.019), while females had increased GAD67-ir compared to males in PPS/SC (p = 0.036), with no significant sex differences in NC/EHC and PPS/EHC. See Figure 5.6.

Basolateral Amygdala (BLA)

There was also a significant prenatal x postnatal x sex interaction in the BLA, $F(1,27) = 10.49$, $p = 0.001$, $\eta_p^2 = 0.434$ (See Figure 5.7). Further analyses were first performed for each sex separately. In males, a significant interaction between prenatal treatment and postnatal treatment was found ($p=0.031$, $\eta_p^2 = 0.273$) and further posthocs determined that PPS/SC had significantly lower levels of GAD67-ir than NC/SC ($p = 0.022$) and PPS/EHC ($p = 0.044$) males. In females, a significant interaction between prenatal and postnatal treatments ($p=0.003$, $\eta_p^2 = 0.542$) was found. Posthoc analyses revealed that NC/SC had significantly less GAD67-ir compared to both PPS/SC ($p = 0.006$) and NC/EHC ($p = 0.037$). PPS/SC females also had significantly higher GAD67-ir levels than PPS/EHC ($p = 0.011$). In NC/SC conditions, females had significantly lower GAD67-ir levels than their counterpart males ($p=0.005$), while a trend existed for PPS/SC females to have higher GAD67-ir compared to males ($p = 0.063$, $\eta_p^2 = 0.488$) while PPS/EHC females had significantly lower levels than their male counterparts ($p=0.007$). See Figure 5.7.

5.4. Discussion

Overall, the effects of prenatal predator stress (PPS) and enhanced housing condition (EHC) were sex- and region- specific. In males, predator stress acted primarily to decrease GAD67-ir density, notably in the PFC and the amygdala (both BLA and CeA). Interestingly, though postnatal EHC had very few effects in naive control (NC) males, only decreasing GAD67-ir in the CeA, the combination of PPS/EHC prevented all of the effects of PPS to decrease GAD67-ir in PPS animals given standard cage (SC) housing, suggesting that GAD67-ir increased in EHC only after being 'triggered' by changes following PPS. Further, the combination of PPS/EHC also increased GAD67-ir

in the male PVN, where neither treatment alone had a significant impact, suggesting PPS is again somehow triggering effects of EHC to increase GAD67-ir in males. In females, PPS/SC offspring had significantly increased GAD67-ir in amygdala (both BLA and CeA), demonstrating an effect of PPS in the opposite direction to that seen in males. PPS/SC females also showed an increase in GAD67-ir in the PVN, where no effects were found in males. Unlike males, exposure to EHC had effects in both naïve and PPS animals, decreasing GAD67-ir in the PFC and NAC-C across both SC and PPS prenatal treatments, which suggests that EHC in these regions was acting independently, not as a compensatory mechanism triggered by PPS. Further, effects of EHC in these regions were in the opposite direction to the compensatory increases in GAD67-ir found in males. However, the direction of effects was again dependent on the region being studied - in the BLA, females given either PPS/SC or NC/EHC showed increased levels of GAD67-ir (again, the opposite direction from males), but the combination of PPS/EHC returned levels to NC levels, suggesting a compensatory mechanism for EHC in this region in the opposite direction of the compensatory increases found in males. Significant sex differences in GAD67-ir were also found in naïve control animals in the NAc-C and the amygdala (both BLA and CeA), with females having less GAD67-ir than males, though after prenatal predator stress, males tended to have less GAD67-ir than females in the PVN and amygdala (both BLA and CeA), suggesting underlying sex differences in these regions during typical development, as well as in response to stress. These interactions between stress, housing, and sex in each region will be discussed in detail below (See Table 5.1 for a summary of effects).

PFC

In males, but not females, PPS decreased levels of GAD67-ir, and these effects were prevented/rescued by EHC in the mPFC, though EHC showed no effects in prenatally naïve animals. Previous studies have demonstrated a similar direction of change (decreased GAD67-ir) in the frontal cortex following prenatal restraint stress in male mice at PND0 (Stevens et al., 2012) or PND21 (Uchida et al., 2014), as well as in adulthood after prenatal dexamethasone treatment (Cerqueira et al., 2005). These decreases in GAD67-ir after prenatal stress have been linked to disrupted migration during embryonic development (Stevens et al., 2012), as well as reduced neurogenesis (Uchida et al., 2014) and neuronal atrophy/loss (Cerqueira et al., 2005) in GABAergic interneurons in the PFC. Notably, all of these studies used male mice and rats, and parallel our findings with PND15 male rats. The fact that we do not see this same decrease following PPS in PND15 females highlights the importance of including both sexes in studies of the effects of prenatal stress on development, and further research is required to examine what is how/why females are less sensitive to the effects of prenatal stress on GABA at this time point.

Interestingly, prenatal and postnatal maternal EE has previously been found to increase concentrations of GABA in the PFC rats at PND42 in both males and females (Connors et al., 2015), which partially mirrors our findings of EHC 'rescuing' the effects of PPS in male pups. However, in our case, EHC only increased GAD67-ir in males after exposure to prenatal stress, which suggests an interesting interaction between these prenatal and postnatal processes during early development. Further, in our study the effects of EHC in females were in the opposite direction of males, and occurred regardless of prenatal condition, suggesting intriguing differences in the effects of the

early environment on mPFC GABA between the sexes. The opposite direction of effects of EHC in females, and the lack of effect of EHC alone in males could be attributed to a number of differences between the two study paradigms, as the authors measured GABA concentrations rather than density of cells, measured at a post-pubertal time point, and using a model of EE that differed both in type and duration to ours (Connors et al., 2015). A more thorough characterization of GAD67-ir ontogeny across early life could help identify sex differences in the developmental trajectory of this region, both during normal sexual differentiation and in response to stress or EHC, and may help clarify the nature of these sex differences in vulnerability to early life interventions.

The medial PFC is believed to be a key brain region involved in both the regulation of the stress response, and in neuropsychiatric disorders (reviewed in McLaughlin et al., 2014). Some clinical studies have suggested that reductions in GABA are important in particularly important in anxiety or panic disorders (reviewed in Domschke and Zwanzger, 2008). In addition to the decreased mPFC GAD67-ir in juvenile males, we have also reported an increase in open field anxiety behaviours in adult male, but not female rats, and these behavioural changes were partially rescued by EHC (Green and Perrot, unpublished data, Chapter 2), suggesting a potential role for the disruption of early life GABA in mediating the adult male anxiety behaviours observed. However, if decreased GAD67-ir in the mPFC were associated with increased anxiety behaviour in both sexes, we would also have expected to see similar anxiety behaviours following EHC in females, and we reported no such effects. Instead, EHC appeared to rescue some aspects of depressive behaviours in juvenile and adult females (Green and Perrot, unpublished data, Chapter 2), and, given the role of the PFC in depressive

behaviours, we could also interpret this finding as suggesting that decreased GAD67-ir in the juvenile female PFC relates to improved adult depressive behaviours. Interestingly, some clinical research suggests that the dlPFC may play a distinct role in depressive behaviours in men and women (Carlson et al., 2015), which highlights the possibility that our sex differences are reflecting different functional roles for GABAergic neurons in the PFC in anxiety/depressive behaviours of males and females. Supporting this idea of different functional circuits in male and female brains underlying anxiety/depressive behaviours, knocking out GR mediated feedback in forebrain regions (PFC, hippocampus, amygdala) results in altered corticosterone responses to acute stress, as well as increased depressive behaviours in the FST and sucrose preference task, in male, but not female mice (Solomon et al., 2012), suggesting that forebrain GR feedback may be more/differentially important in males than females in this model, though the importance of sex differences in GABA in these feedback different circuits remains to be investigated.

Though changes in the GABAergic system following early life insults are often reported as contributing to later behavioural changes, the direction of effects is sometimes contradictory in animal models, with studies reporting both increases and decreases in GAD67 and receptor subunits following early life stress. Further, these effects are often sex-specific, suggesting a complex role for GABA in mediating behaviour (León Rodríguez and Dueñas, 2013; Uchida et al., 2014; Gunn et al., 2015; Labouesse et al., 2015; Luscher and Fuchs, 2015; Li et al., 2015b). In this study, we examined overall density of GAD67-ir as a marker of GABAergic cells. It is possible that different subtypes of GABAergic neurons could be affected in males and females by our early life

interventions (Leussis et al., 2012; Zuloaga et al., 2012a), or that other aspects of the GABA system; the composition of receptor subunits, for example (León Rodríguez and Dueñas, 2013), or changes in dendritic branching of GABAergic cells, could be altered in ways that are not reflected by overall GAD67-ir. Alternatively, it may be that changes in overall GAD67-ir are present, but not reflected at PND15, and only arising at a different developmental time point. Notably, the mPFC continues to develop through adolescence, and to form connections with other areas including the amygdala, PVN and hippocampus throughout early development, and these brain regions are also linked to anxiety/depressive disorders (reviewed in Gunn et al., 2015). During adolescence, there is substantial apoptosis and synaptic pruning in the mPFC, and these changes are highly sex-specific (Hammerslag and Gulley, 2015). Thus, it is possible that our opposite effects of PPS and EHC in PND15 males and females might reflect underlying changes in the developmental time course of GAD67-ir that could be further amplified or disrupted by sex-specific changes during puberty, resulting in the different adult behaviours (anxiety in males and anhedonia in females) we have observed (Green and Perrot, unpublished data, Chapter 2). Supporting this hypothesis, a study in male mice reported that prenatal exposure to an immune challenge resulted in changes in PFC GAD65/67 protein levels that were found in adult, but not peripubertal male mice (Richetto et al., 2014). As this study used a different model of prenatal insult, and only included male subjects, it is difficult to draw direct comparisons, but it does suggest that further research into both other markers and other time points in both sexes are required to investigate these possibilities. Indeed, another recent paper further highlights the importance of including multiple time points and both sexes in prenatal stress research, and has reported that male

and females rats have both sex- and age-specific changes in parvalbumin levels in the PFC following postnatal maternal separation stress; where females demonstrated juvenile, but not adolescent, decreases in parvalbumin protein levels, while in males the decrease in parvalbumin did not emerge until adolescence, and these decreases corresponded to emerging deficits in social interactions (Holland et al., 2014).

Nucleus Accumbens

There were no significant group differences in GAD67 expression in the NAc-Sh, though there was an overall interaction between PPS and sex. In the NAc-C, EHC decreased GAD67-ir in females regardless of stress exposure. The NAc is important for reward processing and motivation, and is a key component of the mesolimbic dopamine system (reviewed in Cartier et al., 2015; Loke et al., 2015). Abnormal NAc development has also been associated with anhedonia in human studies (Wacker et al., 2009), in particular following early life stress (Goff et al., 2013), while prenatal restraint in rats has been linked to sucrose anhedonia in female, but not male offspring (Van den Hove et al., 2014), as have maternal separation paradigms (reviewed in Pizzagalli, 2014). We have also reported a female specific increase in sucrose anhedonia following PPS, that is rescued/prevented by postnatal EHC (Green and Perrot, unpublished data, See Chapter 2). As we reported no significant effects of PPS in the NAc, it is possible that GABAergic changes are not playing a role in anhedonia in our model, or alternatively, that the GABAergic changes relate to other time points, or other aspects of the system not captured in density of GAD67-ir cells. However, the effects of EHC in females, but not males, suggests that decreased GAD67 in the NAc-C could potentially be involved in the 'rescue' of sucrose anhedonia, by independently altering GAD67 density in such a way as

to compensate for some of the effects of PPS in other neurotransmitter systems/brain regions, though further research is required to investigate this possibility.

PVN

As with the mPFC, the effects of PPS and EHC were sex and treatment specific within the PVN. In males, neither PPS nor EHC treatment alone altered GAD67-ir levels, while the combination of PPS/EHC resulted in an increase in GAD67-ir compared to all other groups. Conversely, in females, PPS increased GAD67-ir, and the combination of PPS/EHC returned levels to normal. Interestingly, one recent paper has found that embryonic exposure to a GABA-B receptor antagonist (during a period that partially overlaps the time of our PPS exposure) can alter the rate of movement of estrogen receptor (ER) α positive cells in the PVN in a sex-specific pattern, resulting in a different pattern of localization of cells within the PVN. These changes corresponded to altered anxiety behaviour in females, along with hyperactivity in adult males, and decreased depressive behaviour in both sexes (Stratton et al., 2014). Although we did not examine lateral movement of neurons within the PVN, this study highlights the sex-specific effects of disruptions to GABA in the PVN on anxiety and depressive behaviours. Interestingly, our results in the female PVN again mirror those found in our adult depressive behaviours in females (effects in adult female, but not male, depressive behaviours), but do not correlate clearly with any of the male behaviours observed (Green and Perrot, unpublished data, see Chapter 2), which again suggests possible different neural circuitry at work in these behaviours.

Both clinical and animal studies have suggested that increased CRF in the PVN correlates with anxiety and depressive behaviours (Raadsheer et al., 1994; Blume et al.,

2008; Wang et al., 2008; Zohar and Weinstock, 2011), and some studies link CRF expression with GABAergic inputs (Bowers et al., 1998), suggesting our GAD67 changes could be linked to both an altered stress response and altered CRF-ir. However, we have previously reported that EHC decreased levels of CRF-ir in both males and females on PND15, with no effects of PPS on CRF-ir in the PVN (Korgan et al., 2015). Despite overall sex differences in plasma glucocorticoid levels in PND15 pups, PPS alone also did not appear to increase circulating GCs in either sex (Green and Perrot, unpublished data, See Chapter 2). As the stress-hyporesponsive period is thought to end around PND14 (reviewed in Wiedenmayer et al., 2005; Lupien et al., 2009; Bock and Braun, 2011), the effects of PPS or EHC on GAD67 could be altering aspects of the stress response that are not yet functionally represented in plasma GC levels or CRF-ir at this time point. Supporting this idea, a postnatal immune challenge via LPS on PND3 resulted in changes to CRF mRNA levels in the PVN that did not emerge until PND28 (Amath et al., 2012) – it is possible a similar delayed effect may be at work in our model. Alternatively, as discussed above, it is possible that other GABAergic changes are present at PND15, for example in a certain subtype of GABAergic neurons, or in receptor subunit composition, that are not captured by overall GAD67-ir levels in this study.

Amygdala

In both the BLA and CeA, there were significant sex differences in the density of GAD67-ir in males and females in naïve control animals, with males having a greater density of GAD67-ir than females. Both the BLA and CeA also showed significant sex-specific effects of PPS treatment, where PPS deceased GAD67-ir in males, and increased GAD67-ir in females. Despite the opposite effects of PPS, both males and females

showed a ‘rescue’ by EHC, with the combination of PPS/EHC returning levels to those of NC/SC, regardless of direction of change. These results are in contrast to studies of prenatal DEX treatment, where females, but not males, showed a decreased density of calretinin, a calcium-binding protein used as a marker of a subtype of GABAergic cells in the adult amygdala (Zuloaga et al., 2012b), though this effect is in adults, and only one subtype of GABAergic neurons, and may reflect differences in the development of the amygdala over time. Indeed, other studies have found that the effects of prenatal stress on the amygdala (in particular on the developing GABAergic system), only begin to emerge at PND17, PND21 or PND28, depending on the markers studied (Kraszpulski et al., 2006; Brummelte et al., 2007; McLaughlin et al., 2014; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015), suggesting that we can only interpret our findings of altered GAD67-ir density as a marker of disruption to development, not necessarily an indicator of the final adult level/direction of GABAergic changes.

The amygdala plays a well-documented role in both anxiety/fear responses, as well as social behaviours, including juvenile social play (Blackford and Pine, 2012). Interestingly, we have reported that our model of PPS had significant, sex-specific effects on anxiety and social behaviours, decreasing rough and tumble play in male, but not female, peri-pubertal rats, and increasing open field anxiety in adult males, while increasing anhedonia in adult females, but not males (Green and Perrot, unpublished data, Chapter 2). It is possible that these opposite effects of PPS in the male and female amygdala could be linked to these different behaviours, with decreases in GAD67-ir reflecting increased anxiety or reduced social play behaviour in males, and increased GAD67-ir correlating with increased depressive behaviours in females, though more

research is required into the sex-specific nature of these effects. The CeA and the BLA have been implicated in both anxiety and depressive behaviours (Kraszpulski et al., 2006; Laloux et al., 2012; Mcewen et al., 2012; Oliveira et al., 2012; Ehrlich and Rainnie, 2015), and studies suggest that early life insults can significantly alter both amygdala development and expression of juvenile anxiety and depressive behaviours (Zuloaga et al., 2011a; Raineki et al., 2012; Réus et al., 2013b). The amygdala appears to play a key role in the development of normal sex differences in juvenile play behaviour (Auger and Olesen, 2009; Auger et al., 2011; Forbes-Lorman et al., 2012), and we and others have reported that prenatal stress significantly decreases rough and tumble play behaviour in male rats (Green and Perrot, unpublished data, Chapter 2; (Morley-Fletcher et al., 2003; Paul et al., 2014; Berry et al., 2015). Similarly, one recent study has found that in a mouse model of GAD67 knock-down, there are also significant disruptions in normal male social behaviours (Sandhu et al., 2014). Though PPS/EHC rescued the effects of PPS on GAD67-ir in the amygdala in both males and females, it did not rescue the effects on sexually dimorphic rough and tumble play, however other aspects of play (exploration and social rest) were normalized by PPS/EHC in both males and females. Rough and tumble play is associated with masculinizing levels of gonadal hormones in the prenatal and postnatal period (Auger and Olesen, 2009), and prenatal stress has been found to disrupt these perinatal gonadal hormones (Kapoor and Matthews, 2011; Walf and Frye, 2012; Pallarés et al., 2013b), which suggests our effects of PPS on male rough and tumble play could have been due to gonadal hormones, and the 'rescue' provided by EHC was insufficient to counteract these effects. Interestingly, changes in postnatal tactile stimulation (simulating licking and grooming) have been found to have sex-specific

effects on juvenile play behaviour, where increased tactile stimulation decreased juvenile play in males with no effects in females, and this effect was related to 5HT receptors in the amygdala (Edelmann et al., 2013). Though maternal care was not directly examined in this study, other work from our laboratory suggests that EHC may alter some aspects of maternal behaviour, including high arched back nursing and time on the nest (Perrot and Korgan, unpublished data), which suggests that EHC could be altering other aspects of the developing amygdala via changes in maternal behaviour.

5.4.1. Concluding Remarks

Overall, our model of prenatal stress had a number of sex and region specific effects on GAD67-ir in brain regions associated with anxiety, social and depressive behaviours. EHC appeared to prevent the majority of these effects of prenatal stress, though it also had independent effects on GAD67 levels, primarily in females. Though our changes in GAD67-ir did not map perfectly onto our previously reported behavioural changes (Green and Perrot, unpublished data, See Chapter 2), they do suggest that sex-specific changes in developmental GABA could be contributing to the later development of some anxiety/depressive behaviours. This work also highlights the lack of research on developmental GABA using both males and females outside of reproductive behaviours. Notably, our two environmental interventions are not concurrent, with PPS occurring prenatally, and EHC occurring in the postnatal period. Thus, it is possible that PPS and EHC are acting independently on different processes/aspects of GABAergic development, and EHC compensatory mechanisms are rescuing overall GAD67-ir levels without normalizing all of the underlying changes initiated by prenatal GAD disruptions. For example, different subunits of both the GABA-A and GABA-B receptor are

associated with different functional roles, including different aspects of stress resilience, depressive behaviours, and the anxiolytic effects of benzodiazepines (Gassmann and Bettler, 2012; Jacobson-Pick and Richter-Levin, 2012; Shen et al., 2012; Ehrlich et al., 2013; Smith, 2013; O'Leary et al., 2014), and some studies have found that stressors at different times can alter some subunits, but not others (Jacobson-Pick et al., 2008; Laloux et al., 2012), or that juvenile stressors can have different effects on subunits in juveniles and adults, or following a 'second hit' of adult stress (Jacobson-Pick and Richter-Levin, 2012; Jacobson-Pick et al., 2012). This could explain how we are finding effects of EHC sex-specifically 'rescuing' aspects of GAD67 markers, without also observing a complete behavioural reversal in all PPS/EHC behaviours, though some adult PPS behaviours were prevented by postnatal EHC (Green and Perrot, unpublished data, Chapter 2). Alternatively, as discussed above, changes may be occurring at a different developmental time point, in other neurotransmitter systems, or only following a second adult 'hit' of stress, and therefore not captured in GAD67-ir at PND15. A better understanding of how/when our prenatal and postnatal interventions might be disrupting GABAergic development requires more research into the normal development of GAD67-ir, as well as other markers of the GABA system, across other developmental time points, in both males and females.

5.5. Further Studies

The sex and region specific disruptions of our interventions suggest that GAD67-ir is developmentally plastic during the perinatal period, and that this developmental trajectory is sexually dimorphic. In Chapter 6, I will begin to examine the role of gonadal hormones in shaping these sex differences in GAD67 development, by investigating the

impact of normal sex difference, as well as androgens and anti-androgens in establishing any sex differences in GAD67-ir at this same developmental time point (PND15).

TABLE 5.1. SUMMARY OF THE EFFECTS OF PPS AND EHC ON LIMBIC BRAIN REGIONS IN WHICH GAD67-IMMUNOREACTIVITY DENSITY WAS QUANTIFIED.

Brain Region	PPS	EHC	Combination?
PFC	↓ M ≠ F	≠ M ↓F	<> M ≠ F
NAC-C	≠ M ≠F	≠ M ↓ F	≠ M ≠ F
NAC-Sh	≠ M ≠F	≠M ≠ F	≠ M ≠ F
PVN	≠M ↑F	≠ M ≠ F	↑M <> F
BLA	↓ M ↑F	≠M ↑ F	<> M <> F
CEA	↓ M ↑F	≠M ≠ F	<> M <>F

Summary of significant group effects of treatment on offspring GAD67-ir in limbic brain regions by prenatal predator stress (PPS) alone, enhanced housing condition (EHC) alone, or the combination of the treatments.. ↑ or ↓ indicates direction of effects, and < > indicates cases where PPS+EHC rescued/prevented effects of either alone, while ≠ indicates no effects of treatment. M = male, F = female. PFC = prefrontal cortex NAC-C = nucleus accumbens, core, NAC-Sh = nucleus accumbens, shell, PVN = paraventricular nucleus of the hypothalamus, BLA = basolateral amygdala, CEA = central amygdala.

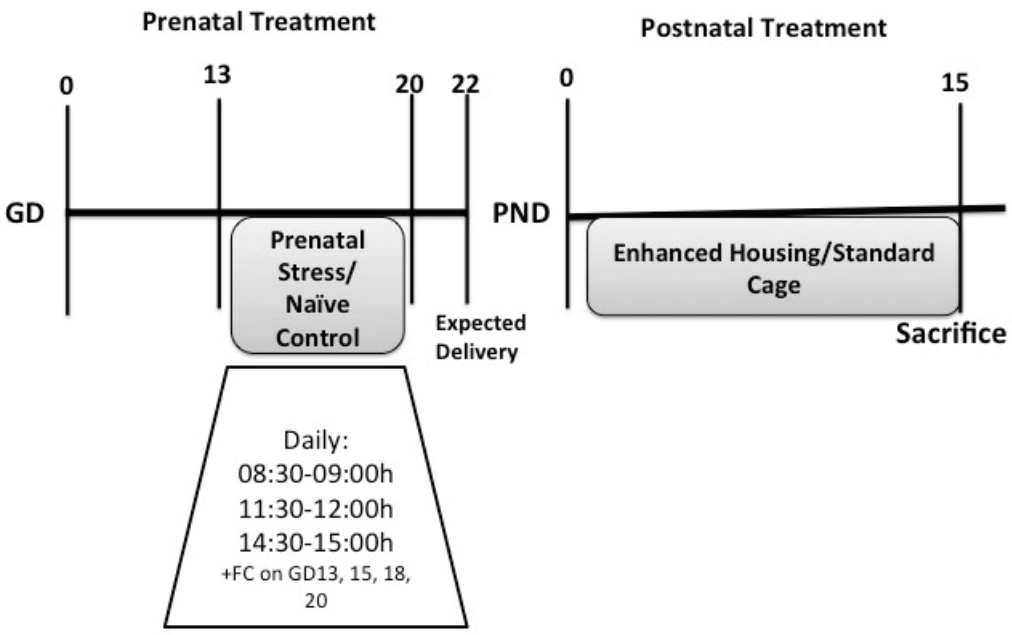

FIGURE 5.1. TIMELINE OF PRENATAL (A) AND POSTNATAL (B) TREATMENT PROCEDURES.

Dams designated to receive prenatal psychological stress (PPS) were exposed three times daily to a predator threat stressor between gestational day (GD) 13 and GD20, along with fecal collection (FC) on GD 13, 15, 18, 20. On the day of birth, postnatal day (PND) 0, dams and pups were transferred to either an Enhanced Housing Cage (EHC), or a clean Standard Cage (SC) and remained undisturbed until sacrifice on PND15.

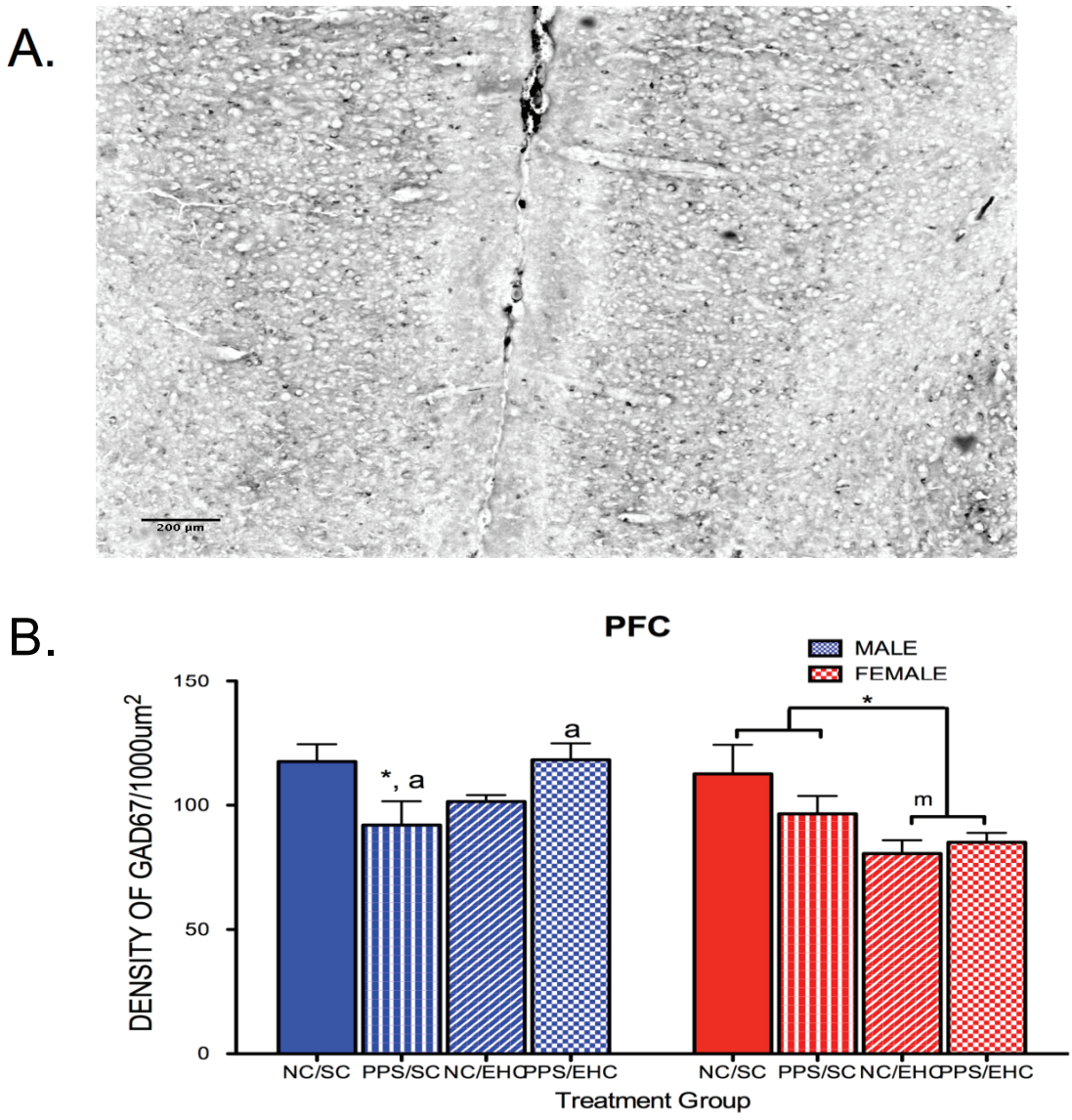

FIGURE 5.2. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE MEDIAL PREFRONTAL CORTEX ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the mPFC at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the mPFC of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed *Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$.

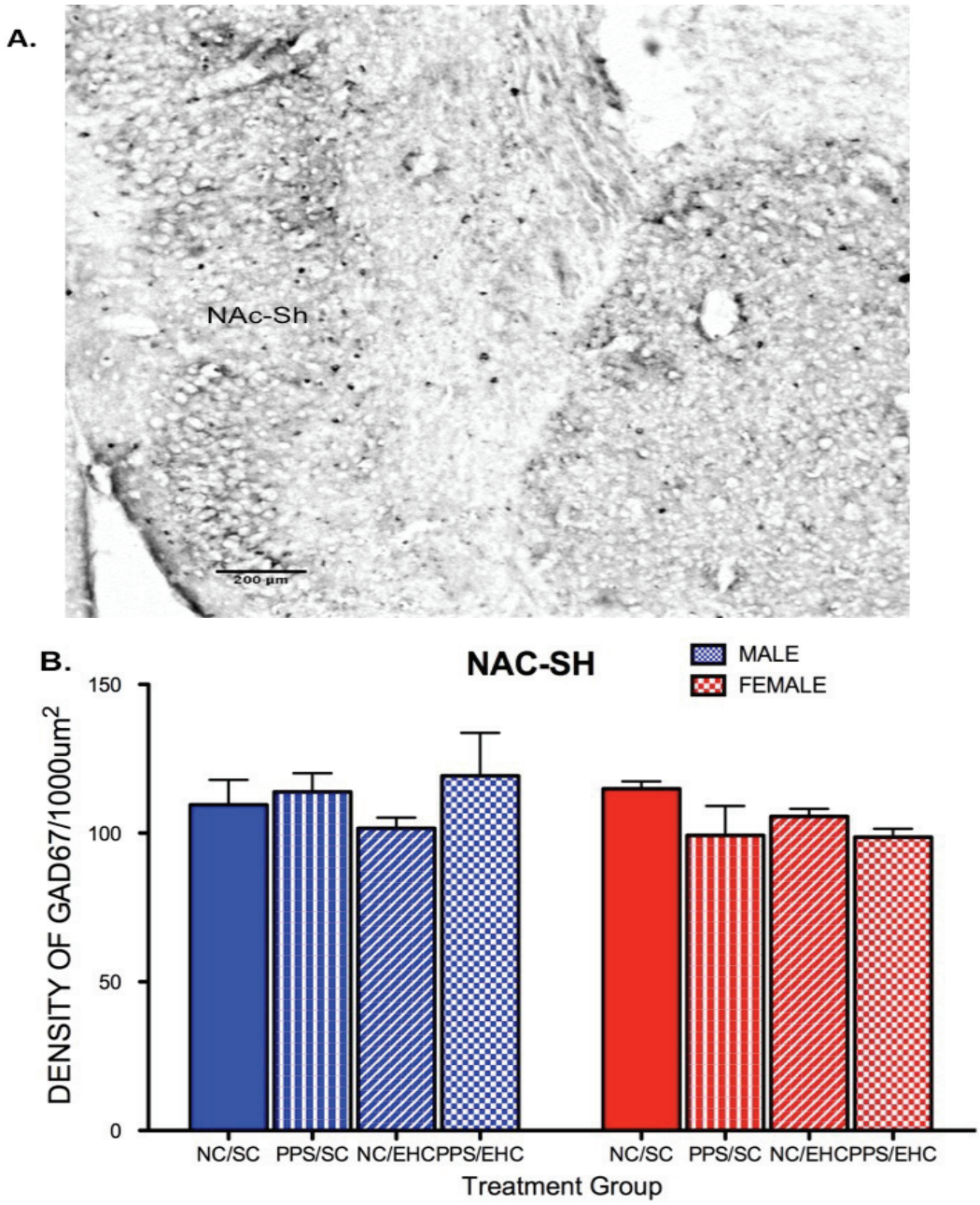

FIGURE 5.3. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE NUCLEUS ACCUMBENS SHELL ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the NAc-Sh at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the NAc-Sh of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed no significant differences.

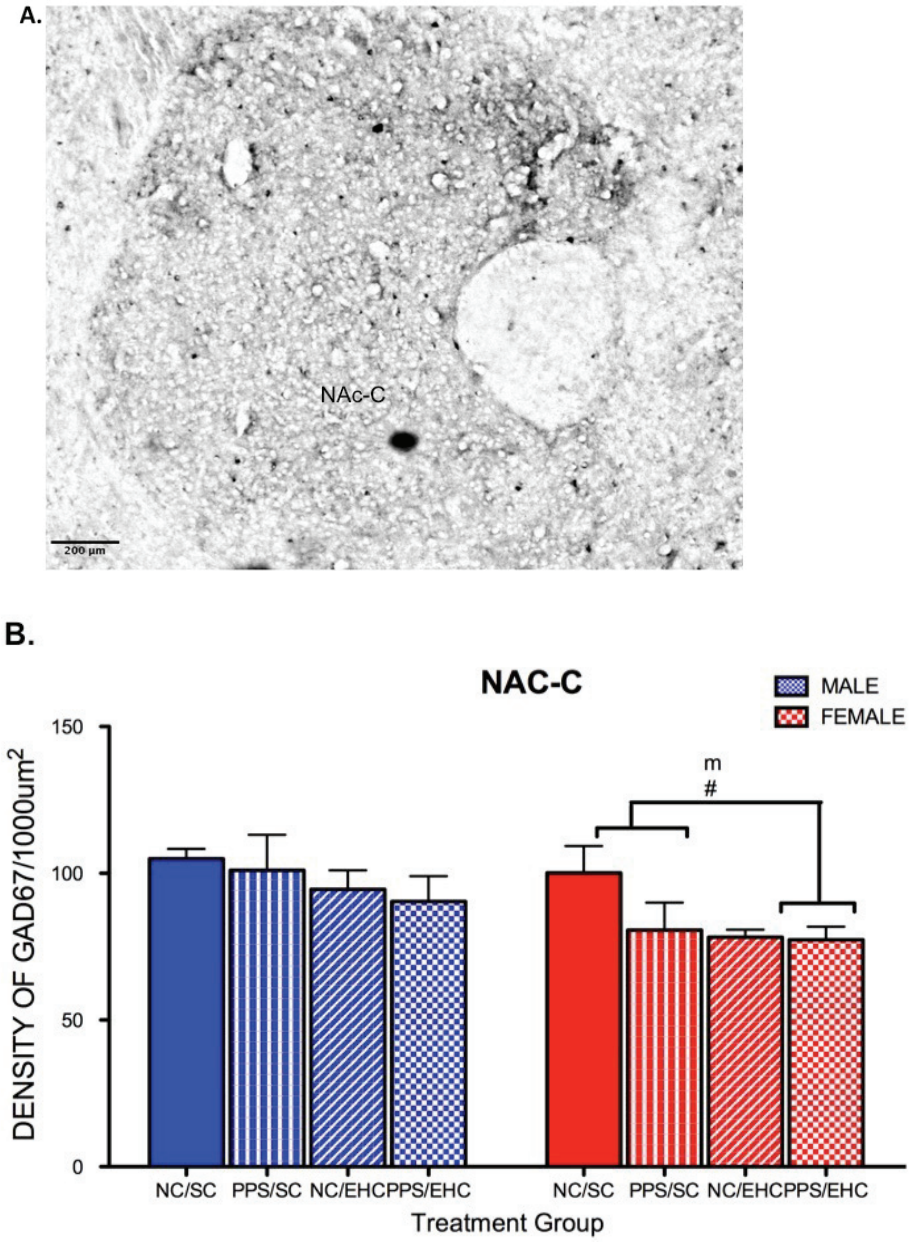

FIGURE 5.4. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE NUCLEUS ACCUMBENS CORE ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the NAc-Sh at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the NAc-Sh of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$.

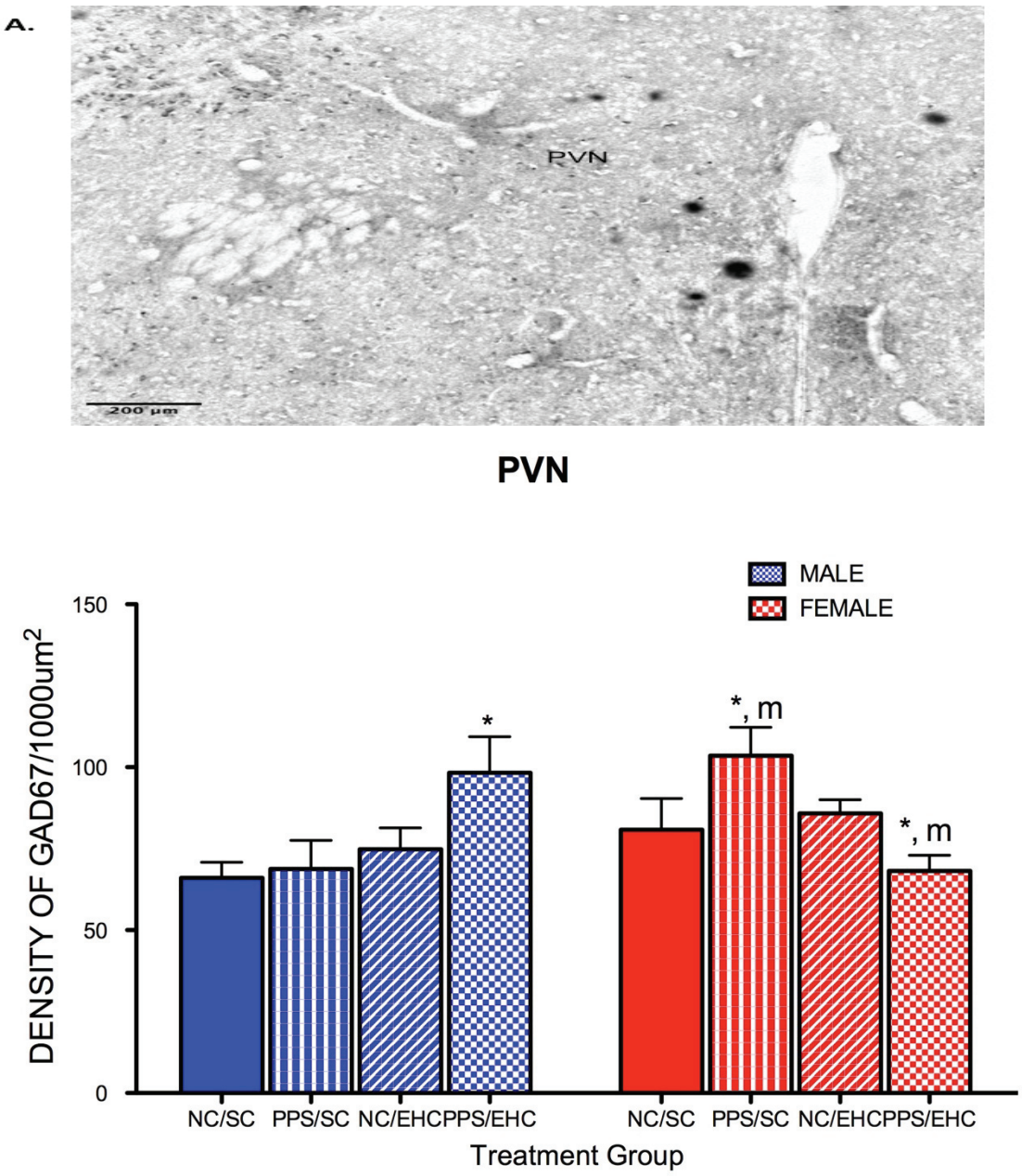

FIGURE 5.5. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the PVN at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the PVN of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed *Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$.

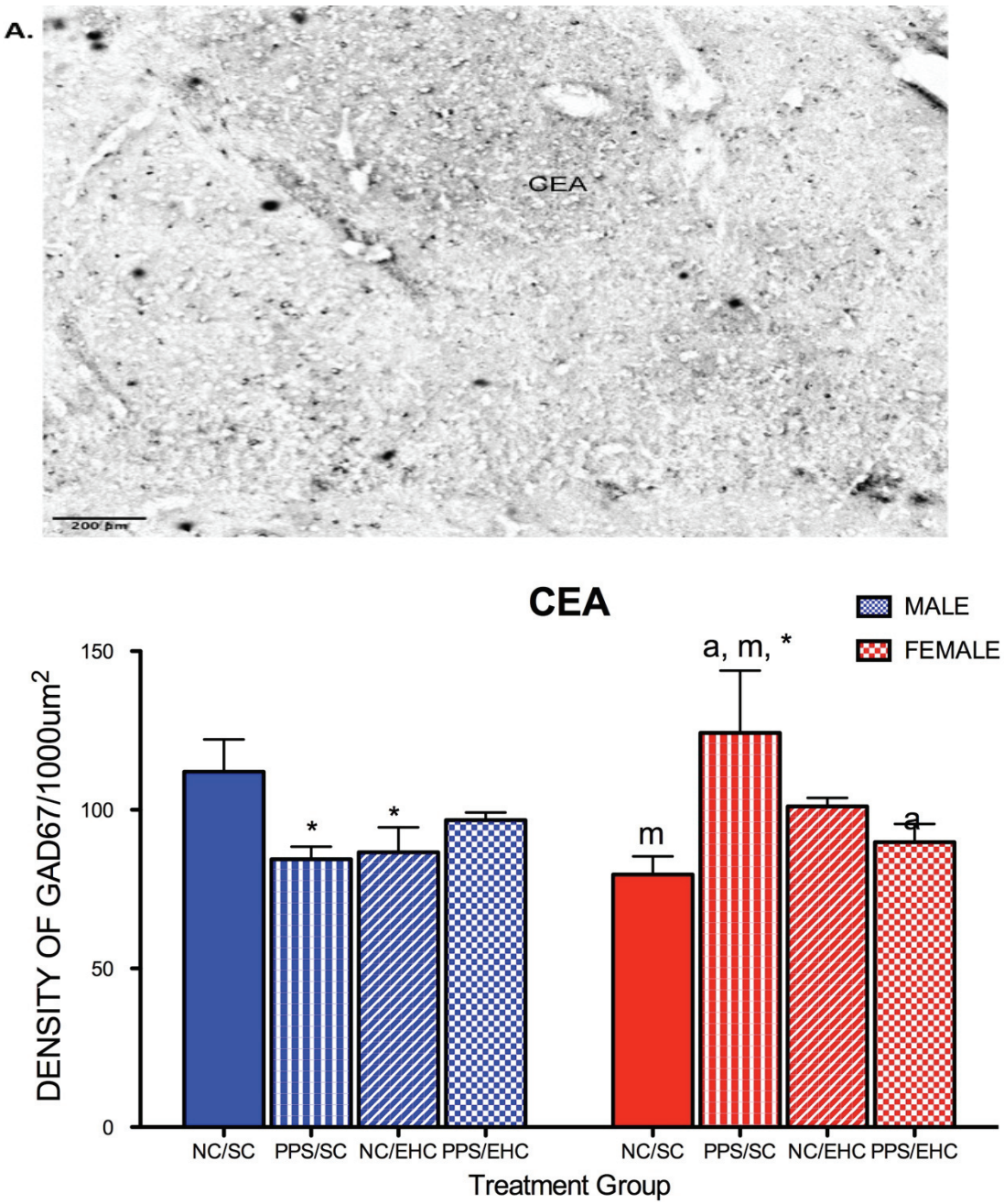

FIGURE 5.6. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE CENTRAL NUCLEUS OF THE AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the CEA at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the CEA of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed *Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$.

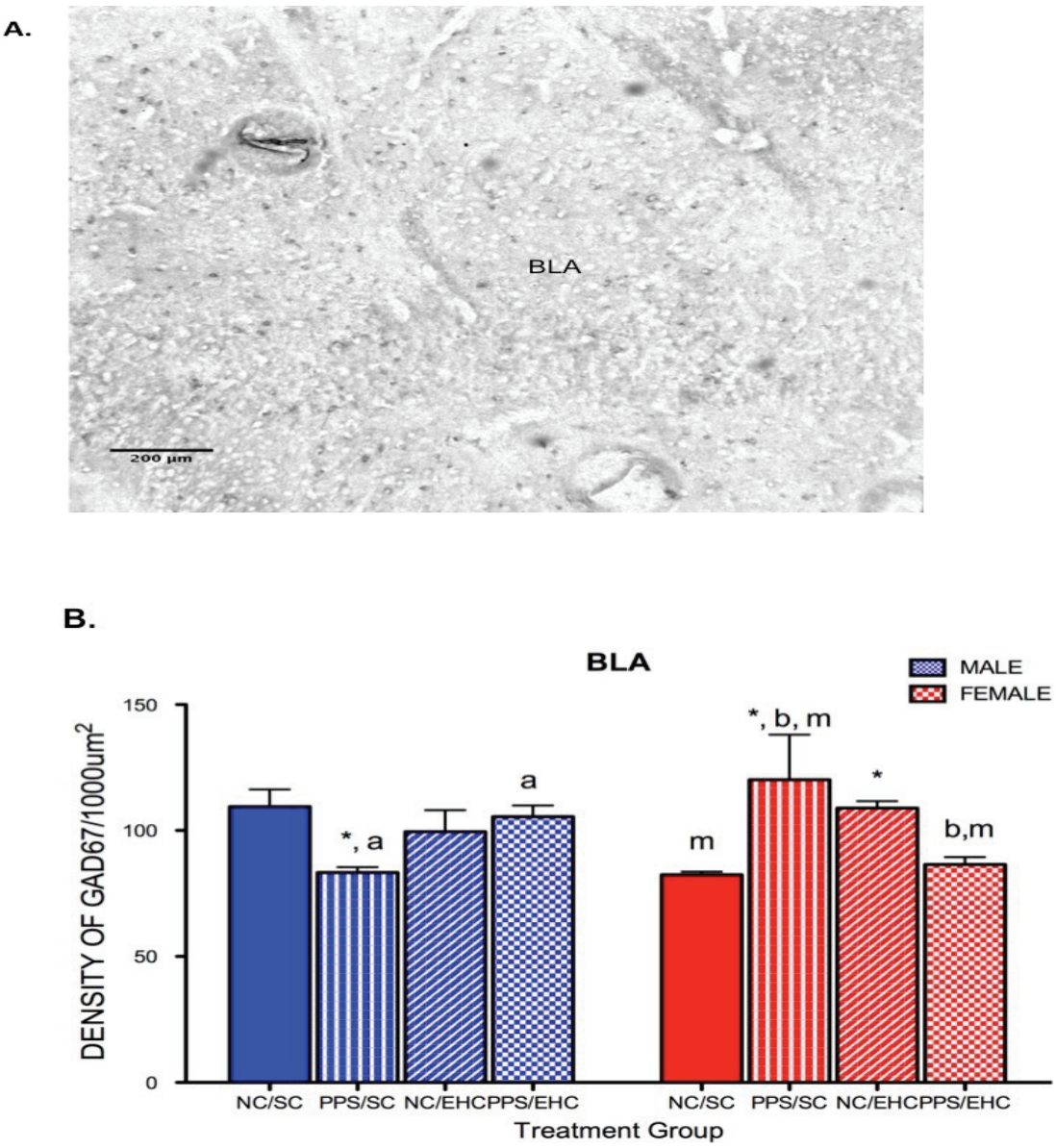

FIGURE 5.7. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE BASOLATERAL NUCLEUS OF THE AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER PRENATAL AND POSTNATAL TREATMENT PROCEDURES.

A. Representative photomicrograph of GAD67-ir in the BLA at 4 x magnification. Scale bar 200µm. Photomicrograph contrast enhanced 0.1%. **B.** Density of GAD67-ir cells in the BLA of postnatal day (PND) 15 male and female offspring that received prenatal psychological stress (PPS) or naïve control (NC) treatment, followed by an Enhanced Housing Cage (EHC), or Standard Cage (SC) postnatally until sacrifice on PND15. Posthoc comparisons revealed *Significantly different from same-sex NC/SC, m– significantly different from males in the same treatment. Bars denoted by the same letters are significantly different from each other, $p < 0.05$.

CHAPTER 6 – THE ROLE OF NEONATAL ANDROGENS IN GABAERGIC CHANGES IN THE JUVENILE BRAIN

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4.1. Introduction

Sex is an important risk factor for the development of a number of neuropsychiatric disorders, including anxiety and depression (reviewed in Altemus et al., 2014), and these differences are due, in part, to underlying biological sex differences (reviewed in Altemus, 2006; Altemus et al., 2014; Bangasser and Valentino, 2014; Loke et al., 2015). Circulating gonadal hormones exert both acute (activational) effects on the brain and behaviour, as well as permanent (organizational) effects when these hormones are present/absent during critical periods of early development. In rodent models, these organizational changes occur during a sensitive period across late gestation and the early postnatal period, when two surges of gonadal testosterone alter trajectories of brain development through their active androgen and estrogen metabolites. These metabolites trigger a number of changes in the brain, altering rates of neurogenesis, neuronal migration, cell death, and synaptogenesis, which contribute to sex differences in the size of many brain regions, as well as the connections between/within these regions (reviewed in Weinstock, 2011; Lenz et al., 2012; Mccarthy et al., 2012).

Much of the focus in animal models has been on the role of organizational hormones in differentiating the brain regions and behaviours underlying reproduction (reviewed in McCarthy, 2007; Mccarthy et al., 2012); however, various non-reproductive behaviours and the underlying neural circuitry are influenced by gonadal hormones and are sexually dimorphic (Giorgi et al., 2014; Goldstein et al., 2014; Bale and Epperson, 2015; Hammerslag and Gulley, 2015; Maeng and Milad, 2015). For example, evidence indicates that organizational hormones are important for both the normal stress response in males and females (McCormick and Mahoney, 1999; Seale et al., 2005b; Gonzalez et

al., 2011; Bingham et al., 2011b; 2012), and for underlying differences in brain regions associated with anxiety/depressive behaviours, including the hippocampus (Zhang et al., 2010a), amygdala (Bingham and Viau, 2008; Auger et al., 2011; Jessen and Auger, 2011), BNST (Garcia-Falgueras et al., 2005; Gotsiridze et al., 2007), prefrontal cortex (Markham et al., 2013) and hypothalamus (Gonzalez et al., 2011). Similarly, results from both human and animal studies suggest that disruptions to prenatal and early postnatal hormone levels may contribute to anxiety or depressive behaviours (Fujimoto et al., 2006; O'Reilly et al., 2010; Tian et al., 2010; Zhang et al., 2010a; Jašarević et al., 2013; Kundakovic et al., 2013; Brown et al., 2015), although the specific underlying biological mechanisms remain elusive. More study of the mechanisms by which early life hormones contribute to biological sex differences in brain regions associated with stress, anxiety, and depression will inevitably aid in characterizing and understanding sex differences in the risk, severity, and presentation of neuropsychiatric disorders as well as provide potential avenues for treatment interventions (Altemus, 2006; Bangasser and Valentino, 2014).

One means for organizational hormones to contribute to sex differences in anxiety or depressive behaviours is through altering developmental expression of the neurotransmitter GABA. In recent years, there has been a strong research interest in the GABAergic deficit hypothesis of depression (Luscher et al., 2011). GABA is omnipresent in the adult brain, where it acts as the principal inhibitory neurotransmitter. GABAergic neurons and their receptors are found in a number of brain regions associated with anxiety/depression and stress responding, including the hippocampus, amygdala, prefrontal cortex, and hypothalamus, as well as modulating the release of other

neurotransmitter systems including dopamine, norepinephrine, and serotonin (reviewed in Croarkin et al., 2011). Clinically, imaging studies have demonstrated reduced GABA transmission (Sanacora et al., 2004; Klumpers et al., 2010) or reduced levels of GABAergic inhibition (Levinson et al., 2010) in depressed patients, and postmortem studies of depressed patients have reported disruptions of GAD67 protein and mRNA levels (Rajkowska et al., 2007; Guilloux et al., 2011; Kang et al., 2012). Moreover, several recent postmortem studies have also noted changes in GABA receptor subtypes following suicide and major depressive disorders (Merali et al., 2004; Poulter et al., 2008; Sequeira et al., 2009; Poulter et al., 2010). Notably, one study reported decreased cortical GABA levels in patients currently in remission, which suggests that GABAergic changes may be a marker of vulnerability to depression, rather than current disease state, making it a particularly interesting target to study developmentally (Bhagwagar et al., 2008). Indeed, recent research using a pediatric population has noted that there is a significant difference in excitatory and inhibitory responses to transcranial magnetic stimulation in clinically depressed children and adolescents compared to healthy controls (Lewis et al., 2016), suggesting disruptions to cortical inhibition could contribute to depressive symptoms in early life.

Results from studies using animal models also point to an important role of early life GABA in the development of later depression or anxiety vulnerability. A conditional mouse knockout of the γ2 subunit of the GABA-A receptor during early postnatal development results in altered HPA axis function, increased anxiety behaviour, as well as altered sucrose consumption anhedonia and forced swim test 'despair' in adulthood (Shen et al., 2010; 2012). Similarly, disrupting GABA-B receptor function by prenatal

injections of a GABA-B receptor antagonist have been found to increase anxiety behaviours in female rats, and decrease depressive behaviours in both male and female rats, as well as blunt the adult HPA axis response to stress (Stratton et al., 2014). Beyond evidence for GABA's involvement in the organizational effects of gonadal hormones on sex differences in anxiety or depressive behaviours (reviewed in Bock et al., 2014), GABAergic disruptions might also underlie some of the effects of stress in early life on later risk of neuropsychiatric disorders (Deslauriers et al., 2013; Uchida et al., 2014; Ehrlich and Rainnie, 2015). Notably, many of these behavioural changes brought on by either early life stress or direct GABAergic disruptions are sexually dimorphic, demonstrating an interesting mechanistic link between GABA dysfunction, stress, and sex differences in depression vulnerability.

As described above, organizational effects of early life gonadal hormones contribute to sex differences in vulnerability to anxiety and depression, either by early life stressors disrupting GABA development in sexually dimorphic ways, or through creation of sex-specific vulnerabilities in 'typical' development. In addition to GABA's role in the response to early life stress, GABA is a key player in sexual differentiation in the brain. It undergoes functional maturation during the late prenatal and early postnatal period when many of the long-term effects of early life stress are also organized, and establishing functional GABAergic circuitry during development is vital for maintaining the typical balance of excitation and inhibition in the brain. Further, disruptions to developmental GABA have been linked to several neurodevelopmental disorders including (reviewed in Chattopadhyaya, 2011; Ramamoorthi and Lin, 2011). Early in development, GABA acts as the primary excitatory neurotransmitter in the brain, and is

believed to be involved in regulating neuronal migration, growth, maturation, and synapse formation (Ganguly et al., 2001). Across the early postnatal period, GABA makes the developmental 'switch' from excitation to inhibition via a shift in the intracellular chloride gradient, mediated by changes in the ratios of chloride co-transporters NKCC1 and KCC2. The precise timing of this shift from excitation to inhibition varies by both brain region and sex, and is important for GABA's role in regulating early developmental circuits, and in forming GABAergic synapses (reviewed in Nuñez and McCarthy, 2007; Galanopoulou, 2008a; Ben-Ari et al., 2012; Le Magueresse and Monyer, 2013).

Many parameters of GABAergic development appear to occur along different developmental timelines in different brain regions, and some of these changes appear to be regulated by early life gonadal hormones. For example, the change in NKCC1/KCC2 ratio that underlies the switch from excitatory to inhibitory GABA occurs at different times in the male and female hippocampus and entorhinal cortex (Nuñez and McCarthy, 2007; Murguía-Castillo et al., 2013), substantia nigra (Galanopoulou et al., 2003), and hypothalamus (Auger et al., 2001; Perrot-Sinal et al., 2007) and these changes appear to be related to early life estradiol and testosterone (reviewed in Galanopoulou, 2008a). Similarly, early life levels of GAD, the rate limiting enzyme for GABA synthesis, are sexually dimorphic in some brain regions, including the CA1 region of the hippocampus, the BNST, and the hypothalamus, and these differences are also linked to early gonadal hormone surges (Davis et al., 1996a; Perrot-Sinal et al., 2001; Polston et al., 2004; Zhou et al., 2005).

The majority of research to date has focused on the role of sexually dimorphic

GABA in the development of the hypothalamus, hippocampus, or substantia nigra. Organizational hormones, however, also have long-term programming effects in a number of brain regions linked to anxiety/depressive disorders; including the PFC (Pallarés et al., 2014; Mogi et al., 2015) and amygdala (Auger et al., 2011; Jessen and Auger, 2011; Kolodkin and Auger, 2011; Cao et al., 2013; Hu et al., 2015), though the developmental role of GABA in these sex differences has not been well-characterized. Calcium-binding proteins parvalbumin, and calbindin, used as markers of GABAergic neuronal subtypes, are sexually dimorphic in the adult guinea pig BLA and CeA (Równiak et al., 2015), while parvalbumin neurons in the adult CeA are also sexually dimorphic, and related to anxiety behaviours, in a rat model (Ravenelle et al., 2014). One study has reported that sex differences in adult GABA in the amygdala are altered by neonatal castration (Stefanova, 1998), while another has shown an effect of prenatal androgens on adult GABA-B receptors in the amygdala (Hu et al., 2015). Further, as described previously, evidence suggests GABA as a key point of disruption in the impact of early life stressors or insults in these brain regions, including the nucleus accumbens (Li et al., 2015b), amygdala (Laloux et al., 2012; Zuloaga et al., 2012b; Ehrlich and Rainnie, 2015; Ehrlich et al., 2015) and PFC (Leussis et al., 2012; Matrisciano et al., 2013; Holland et al., 2014; Uchida et al., 2014; Van den Hove et al., 2014). Some of these changes are sexually dimorphic (Holland et al., 2014; Van den Hove et al., 2014; Marron Fernandez de Velasco et al., 2015), and as prenatal stressors disrupt typical surges of perinatal testosterone (Walf and Frye, 2012; Pallarés et al., 2013a), disruptions to organizational hormones could be contributing to these effects of early life stress (Barrett et al., 2013). Together, then, this research highlights a potential role of early life

sex differences, and thus, organizational hormones, in the developmental trajectory of GABA in these anxiety/depression associated brain regions.

Though much of the existing research has focused on the aromatization of testosterone to estradiol as the primary method of sexual differentiation (reviewed in Lenz et al., 2012), increasingly, it has been suggested that perinatal androgens, as well as estrogens, are active participants in masculinizing the adult HPA axis response and associated limbic brain regions (Zuloaga et al., 2011a; Chen et al., 2014; Hamson et al., 2014), as well as some juvenile anxiety/depressive behaviours (Zhang et al., 2010a). Interestingly, testosterone also plays a role in neonatal seizure sensitivity in male rats (Nuñez and Mccarthy, 2008), and recent research has suggested that it acts to modulate seizure activity in the brain in part though actions of the DHT metabolite androstanediol (5α-androstan-3α,17 β-diol), which acts on GABA-A receptors (Reddy and Jian, 2010). In addition, androgens alter rates of adult synaptogenesis in the PFC and hippocampus, as well as cell survival rates in the hippocampus (reviewed in Celec et al., 2015) and have important modulatory effects in the adult HPA axis (reviewed in Handa and Weiser, 2014), and it is possible that some of these same mechanisms could be active across early life, and contribute to neonatal patterns of GABA development.

To summarize what was presented above, the literature suggests that GABA is highly expressed in the late prenatal and early postnatal period (Ben-Ari et al., 2012), and plays a demonstrated role in both sexual differentiation (McCarthy, 2008) and some anxiety/depressive-linked brain and behavioural changes described above (Luscher and Fuchs, 2015). Therefore, GABA is a leading contender for involvement in the hormone-driven sexual differentiation of non-reproductive brain regions, particularly those

associated with stress/depression. Recent work in our laboratory has demonstrated that, at PND15, there are significant sex differences in seizure behaviour in response to prenatal stress/postnatal enhanced housing, as well as sex-specific changes in fos-B-ir and CRF-ir in limbic brain structures (Korgan et al., 2014; 2015). Recently, I demonstrated that this early life stress and housing manipulation also alters DNMT3a-ir and GAD67-ir density in PND15 rats in a sex- and region-specific manner (Green and Perrot, unpublished data, see Chapter 3 and 5). Further, I have also found effects of organizational androgens in DNMT3a in some of these same brain regions (Green and Perrot, unpublished data, see Chapter 4), and a growing body of evidence suggests GABA may be a prime target for DNMT1 and DNMT3a methylation (Kadriu et al., 2012). In order to investigate the potential role of perinatal androgens in organizing these GABAergic sex differences in the early postnatal brain, I used immunohistochemical analysis of GAD67-ir in the PND15 brains of males and females after early postnatal injections of dihydrotestosterone (DHT) in females (to examine 'masculinizing' influence of androgens) or the anti-androgen flutamide (to block androgen receptor activation) in male pups. PND15 is an intriguing time point to examine initially, as it is consistent with our past work, and it is an age where the critical period for sexual differentiation is believed to have ended in many brain regions (reviewed in Mccarthy and Nugent, 2015; Tsai et al., 2015). In keeping with our previous work, and in light of literature suggesting potential roles of androgens in these regions described above, I have focused on the limbic associated regions including the PFC, NAc-C, NAc-Sh, PVN, CeA and BLA.

6.2. Methods

6.2.1. Animals and Breeding

Please note that all of the animals used in this Chapter are the same dams and offspring used in Chapter 4 to examine androgen effects on DNMT3a-ir (Green and Perrot, unpublished data) - immunohistochemical analyses were performed on a separate series of tissue sections through the same offspring brains. Charles River Canada (St. Constant, Quebec) provided adult male and female Long-Evans rats for breeding. Prior to breeding, animals were left undisturbed for at least one week. A sexually experienced male was placed in the cage of pair-housed females for one week to allow for insemination, then females were re-housed singly (with their litters) for the duration of the experiment. Starting 20 days after the start of the breeding week, females were checked daily for pups at the beginning of the dark cycle. The day of birth was designated PND0, and pups in each litter were counted and sexed prior to injections. All males and females were housed in standard colony rooms with wire mesh lids, Plexiglas cages (22 x 21 x 44 cm), pine shavings (Hefler Forest Products Inc., Sackville, NS, Canada) and a black polyvinyl-carbonate (PVC) tube for enrichment. The colony rooms were maintained on a reversed 12:12 hour light:dark cycle with lights off at 09:00h, at 21±2°C. Access to food (Purina Lab Chow) and tap water were provided *ad libitum*. All experimental procedures were pre-approved by the Dalhousie University Committee on Laboratory Animals (UCLA) and were in accordance with the guidelines stipulated by the Canadian Council on Animal Care. An effort was made to use the minimum number of animals required for statistical comparison and to minimize pain and suffering in experimental subjects.

6.2.2. Postnatal Hormone Manipulations

The experimental timeline for postnatal injections is described in Figure 6.1. On PND0, pups were briefly removed from the dam to be sexed and counted, and placed in a clean cage. Pups received subcutaneous (s.c.) injections into the skin on the back of the neck using a 27g needle. Starting on PND0, male pups were assigned to receive s.c. injections of either flutamide or vehicle, on PND0, PND1, then every other day until PND15, with 1 pup from each litter in each group. Female pups received s.c. vehicle or dihydrotestosterone (DHT), on PND0 and PND1, with at least one pup from each litter in each group. All females then received s.c. vehicle injections every other day until PND15. On PND0 all pups were also injected with 1µl of India ink into the left or right hindpaw or forepaw to indicate treatment group. Flutamide is a potent anti-androgen that inhibits androgen uptake and nuclear binding, while DHT is a potent androgen hormone (Konkle and Mccarthy, 2011). Drug doses per injection were as follows: 50µl vehicle (5% ethanol in sunflower oil), flutamide (50 mg/kg), or DHT (100µg/50µl). These doses were chosen as they have been shown to prevent typical sexual behavior and to alter the levels of CRH and GR mRNA in the adult male rat (Seale et al., 2005b), as well as alter prepubertal male anxiety and depressive behaviours (Zhang et al., 2008) and to masculinize sexual behaviour, and alter the levels of CRH and GR mRNA of adult female rats (Seale et al., 2005a).

6.2.3. Sacrifice and Tissue Generation

Two male and two female rats from each litter (one hormone treated and one vehicle control) were sacrificed on PND15 via a lethal dose of Euthanyl (sodium pentobarbital), injected intraperitoneally (i.p.), and brains were processed for

immunohistochemistry. Remaining pups were collected for a separate experiment.

6.2.4. Tissue Preparation and Immunohistochemistry

Following Euthanyl anesthesia, pups were perfused transcardially with 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by 4% paraformaldahyde (PFA) in PBS. Brains were removed from the skulls and post-fixed overnight in PFA, then cryo-protected in 30% sucrose in 1M PBS for 2-3 days before being snap frozen using dry ice cooled isopentane, and preserved at -80°C until slicing. A cryostat was used to obtain serial coronal sections (12 μm) throughout the brain, which were then mounted on double-subbed gelatin coated slides, allowed to dry overnight, and stored at -80°C until processed for IHC. Tissues from 4-5 rats per treatment group and sex were collected for analysis.

Slide-mounted tissue was brought to room temperature over 1 hour, and slides were subjected to Heat-Induced Epitope Retrieval (HIER) in 10mM sodium citrate buffer, pH 6.0, for 15 min at 95°C and allowed to cool in the buffer for 10 minutes. Slides were washed in 3 x 10 minute rinses in PBS, then incubated for 45 min with agitation at room temperature in 0.1% PBS-Tween containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.03% H_2O_2 to inactivate endogenous peroxidases. Following rinses (PBS, 3 x 5 min), sections were incubated overnight with agitation at 4°C with mouse anti-GAD67 (MAB5406, EMD Millipore; 1:1000) diluted in PBS with 1% BSA. Slides were rinsed (0.1% PBS-Tween, 3 x 5 min) and incubated for 90 min at room temperature with biotinylated secondary antibody (KPL, Gaithersburg, Maryland) diluted 1/500 in PBS, rinsed 3 x 5 minute (0.1% PBS-Tween), then incubated for 90 min at room temperature in ABC complex (Vectastain, Vector Laboratories, Burlington, Ontario).

After rinsing (PBS, 3 x 5 min), visualization was achieved using 0.05% diaminobenzidine diluted in PBS + 0.003% H_2O_2. Sections were rinsed (PBS, 2 x 10 min), dehydrated in a graded ethanol series, immersed in Histoclear (Sigma) and cover-slipped using Permount (Fisher Scientific). Both positive and negative tissue type controls were performed, as well as a no primary antibody control IHC to confirm staining effectiveness.

6.2.5. Tissue Analysis

All slides were examined using an Olympus BX43 light microscope (Olympus, Markham, ON) with a 4x and 10x objective and an Olympus XM10 digital CCD camera (Olympus), controlled by cellSens imaging software, with exposure time, brightness, and contrast kept constant for all image captures. A series of every 6^{th} section through several brain regions were used, with a minimum of 4 sections per brain region in each animal (n = 4-5 per group). The series of sections from each brain were selected with reference to an anatomical rat brain atlas (Paxinos and Watson) to define the boundaries of the PFC, NAc-C, NAc-Sh, PVN, BLA, CeA (See Table 3.1 for list of abbreviations). All GAD67-ir neurons (defined as showing a round/oval shape and primarily cytoplasmic staining patterns) present on each section were manually quantified in bilateral sections captured in greyscale images captured under a 10x objective by an experimenter blind to the treatment group and sex of the animals. (μm^2) of each region was calculated on tracings of each section using cellSens and Image J software (NIH, Bethesda, MD, USA). Densities were calculated as a function of the number of GAD67-ir cells relative to the region's area, and data were expressed as the mean number of positive cells per 1000μm^2 of surface area. Sections with large tears or holes in the tissue were not counted.

All GAD67-ir neurons present on each section were quantified bilaterally from captured microscope images using Image J software (NIH, Bethesda, MD, USA).

6.2.6. Statistical Analysis

Differences in GAD67-ir were assessed initially in each brain region using a 2 x 2 ANOVA with treatment (DHT or flutamide compared to vehicle) and sex (male or female) as the between-subject factors. In cases of significant main effects or interactions, appropriate post-hoc tests were performed with a Sidak correction factor. In all cases, an α level of 0.05 was considered an acceptable error level. All analyses were performed in SPSS software (v23, SPSS Inc.).

6.3.1. Prefrontal Cortex

ANOVA analyses revealed no interactions or main effects of sex or treatment $F(1, 13) = 0.194$, $p = 0.667$, $\eta_p^2 = 0.015$. See Figure 6.2.

6.3.2. Nucleus Accumbens

Nucleus Accumbens Shell (NAc-Sh)

ANOVA analyses revealed no interactions or main effects of sex or treatment, $F(1, 13) = 0.17$, $p = 0.897$, $\eta_p^2 = 0.015$. See Figure 6.3.

Nucleus Accumbens Core (NAc-C)

ANOVA analyses revealed no interactions or main effects of sex or treatment, $F(1, 13) = 0.501$, $p = 0.492$, $\eta_p^2 = 0.037$. See Figure 6.3.

6.3.3. PVN

ANOVA analyses revealed a trend towards an interaction between sex and treatment $F(1, 12) = 3.176$, $p = 0.063$, $\eta_p^2 = 0.197$, as well as a main effect of sex $F(1,12)$

= 5.825, p = 0.031, η_p^2 =0.197 (See Figure 6.4). Posthoc analyses revealed this was driven by a trend towards vehicle females having higher levels of GAD67-ir than vehicle males (p = 0.055, η_p^2 =0.481).

6.3.4. Amygdala

Central Nucleus of the Amygdala (CEA)

ANOVA revealed a significant interaction between sex and treatment F(1,12) = 30.905, p = 0.001, η_p^2 =0.72 (See Figure 6.5). Posthoc analyses revealed that both vehicle treated males (p = 0.009) and DHT females (p = 0.001) had significantly higher levels of GAD67-ir than vehicle females, and DHT treated females were also significantly higher than flutamide treated males (p = 0.009).

Basolateral Amygdala (BLA)

ANOVA revealed a significant interaction between sex and treatment, F(1,12) = 26.43, p < 0.001, η_p^2 =0.829 (See Figure 6.5). Post hoc analyses revealed that vehicle treated males had significantly higher levels of GAD67-ir than vehicle females (p = 0.01). DHT treated females had significantly higher levels of GAD67-ir than all other groups (all p's ≤ 0.004). See Figure 6.5.

6.4. Discussion

Overall, GAD67-ir density was significantly different between vehicle treated males and females only in the amygdala (both CeA and BLA), though there was also a trend towards a difference in the PVN. In the PVN, both flutamide treatment and DHT resulted in intermediate levels of GAD67-ir that were not significantly different from vehicle-treated males or females. Blocking the effects of androgen receptors in males using flutamide resulted in levels of GAD67-ir in both the BLA and CeA that were not

significantly different from either vehicle males or females, suggesting that flutamide alone did not completely feminize GAD67-ir in these areas. Treatment with DHT significantly increased levels of GAD67-ir compared to vehicle treated females in both the BLA and CeA. Indeed, in the BLA, levels of GAD67-ir were also significantly higher in DHT females than in vehicle treated males. Thus, the effects of androgens on GAD67-ir in appear to be region specific, and somewhat complex – the particular role of androgens in each of these different regions will be discussed below (see Table 6.1 for a summary of results).

The PVN is the final neural component involved in HPA axis activation and is a key structure in regulating the physiological response to stress. It is surrounded by GABAergic neurons and contains a high density of GABA receptors. As described previously, disruptions to the HPA axis have been linked to anxiety/depressive disorders (reviewed in McClellan et al., 2010). In the PVN, there was a trend towards females having higher levels of GAD67-ir than males, and either treating males with flutamide or females with DHT, resulted in levels that were not significantly different from either males or females. This suggests that postnatal androgens may be involved in partially mediating the sex difference in GAD67-ir, but they do not completely explain the difference. Although it is difficult to extrapolate based on a statistical trend, the effect size was small/moderate, and this finding does support existing research showing that early life gonadal hormones play an important role in adult HPA function (McCormick and Mahoney, 1999; Bingham and Viau, 2008). Interestingly, one recent paper noted that embryonic exposure to a GABA-B receptor antagonist disrupts the prenatal migration of estrogen receptor (ER) α positive cells in the PVN in a sex-specific pattern, which

corresponded to increased adult anxiety behaviour in females, along with hyperactivity in adult males, and decreased depressive behaviour in both sexes (Stratton et al., 2014), supporting the potential role of GABA in the PVN in organizing sex differences in adult vulnerability to anxiety/depression. However, the results of this study also highlight the importance of *prenatal* GABA, and likely prenatal organizational hormones, in the development of this brain region. As described previously, there are typically two surges of gonadal testosterone release, one at approximately GD18, and another within a few hours of birth on PND0 (reviewed in Ward et al., 2002; 2003). Therefore, the intermediate effects of our hormonal treatments could be due to our postnatal flutamide/DHT treatments only targeting one of these periods where organizational hormones could be acting. Alternatively, the literature suggests an important role of both androgens and estrogens in PVN development, as treatment with either an aromatase inhibitor (1,4,6-androstatriene-3,17-dione; ATD) or flutamide, across GD13-PND20 alters levels of CRF, and GR, in the adult male PVN (Seale et al., 2005b). Supporting a role for both androgens and estrogens, male pups given either postnatal ATD or flutamide from PND0-PND21 also failed to show disruptions of HPA axis activity, or the usual habituation in plasma corticosterone following repeated stress in adulthood (Bingham et al., 2011). Our findings support this role of postnatal androgens in (partially) shaping the development of the PVN, and highlight the important role of these hormones in organizing sex differences in GABA in this period.

In both the BLA and CEA, males had a higher density of GAD67-ir than females while treatment with flutamide resulted in an intermediate level of GAD67-ir, between that of males and females, in both regions. These results are consistent with previous

studies that have reported significant sex differences in GABAergic neurons in the amygdala (Stefanova, 1998; Ravenelle et al., 2014; Równiak et al., 2015). However, in past studies of adult rodents, the direction of the effect was opposite to that reported in our juvenile animals (females had a greater density of GABAergic neurons than males) though one study focused only on a subtype of parvalbum + GABAergic neurons (Ravenelle et al., 2014). The amygdala continues to develop throughout the juvenile and adolescent period, and the absolute number of GABAergic neurons likely continues to change throughout this period. Indeed, one recent study in the dorsal hippocampus found a gradual increase in female, but not male, parvalbumin+ cells across the juvenile and adolescent periods. This increase in GABAergic cells appeared to be mediated by pubertal estrogens, as adolescent ovariectomy prevented this increase, and the effect was rescued by estradiol replacement (Wu et al., 2014). Though further research is required, it is possible that a similar process occurs in the amygdala, with females 'overtaking' males in the density of GABAergic cells by adulthood. We were able to find one study in PND20 rats that examined the cortical amygdala, and found a sex difference in GAD67-ir, which is in line with the direction of our sex difference in the BLA and CEA (males > females; Stefanova et al., 1997), while the same group reported the opposite direction of effects in adulthood (females > males; Stefanova, 1998). This research supports this idea of a later (adult) reversal in the direction of the sex difference in GAD67-ir in these regions, with females 'overtaking' males in GAD67-ir density in adulthood. As we have observed a significant difference in GAD67-ir on PND15 that is partially mediated by androgens, organizational exposure to gonadal hormones could prime these differences in developmental trajectory, triggering these later changes, and resulting in different

adolescent/adult amygdala circuitry. Given the importance of the amygdala for social development (Blackford and Pine, 2012), the importance of GABAergic markers in the amygdala for expression of anxiety and depressive behaviours (Zuloaga et al., 2011a; Raineki et al., 2012; Réus et al., 2013a; Ravenelle et al., 2014), and our own research pointing to an emergence of sex-specific anxiety/depressive behaviours only after adolescence (Green and Perrot, unpublished data, see Chapter 2), this appears to be a particularly intriguing avenue of future investigation.

In both the BLA and CEA, flutamide only partially feminized the sex difference in GAD67-ir density. There are several possible explanations for this finding. First, postnatal estrogens may be partially responsible for the sex difference found in the BLA and CEA, and given the driving role of estrogen in organizational sex differences in other brain regions (reviewed in McCarthy, 2008), and in organizing juvenile play behaviour via the amygdala (Kolodkin and Auger, 2011) the role of estrogens in this difference is an important possibility to investigate in the future. Further supporting this idea, neonatal castration, which would remove both gonadal estrogens and androgens, prevents the sex difference in GAD67-ir in the adult BLA (Stefanova, 1998). As discussed previously, our postnatal hormone intervention is only targeting one of the two surges in testosterone released by the testes (reviewed in Ward et al., 2002; 2003), and the partial effects of our androgen antagonist could also be due to the effects of prenatal androgens/estrogens. Supporting this idea, prenatal testosterone has been reported to alter the expression of GABA-B receptors in the adult amygdala, suggesting that these receptors are at least somewhat involved in normal GABAergic development in the amygdala (Hu et al., 2015). Further, sex chromosome complement may also be playing an important role in

regulating GABAergic development. A recent study has found that adult XY mice have significantly lower levels of GABAergic gene expression, including GAD67, than XX mice, and gonadal sex only partially mediated some of these GABAergic gene differences, with complex gene-hormone interactions on anxiety behaviours (Seney et al., 2013).

Given the incomplete effects of flutamide, the effects of postnatal DHT on the amygdala of female rats were somewhat surprising, as DHT completely masculinized levels of GAD67-ir in the CEA, and increased the density of GAD67-ir in the BLA to levels even greater than those found in vehicle treated males. It is important to note here that, although DHT is considered the most potent androgen receptor activating metabolite of testosterone, it can itself be further metabolized in the brain. DHT can be converted to 5α-androstane-3β,17β-diol (3βAdiol) or 5α-androstane-3α,17β-diol (androstanediol) by other steroidogenesis enzymes in the brain. Interestingly, it has recently been demonstrated that androstanediol itself is a modulator of GABA-A receptors, and may have direct actions on GABA-A receptors at high concentrations (Reddy and Jian, 2010), while 3βAdiol has been shown to act at an estrogen-response element (ERE) within the estrogen receptor β (ERβ; Lund et al., 2006; Pak et al., 2007) gene. Thus, it is possible that, in addition to the effects of DHT on androgen receptors, DHT metabolites could also be acting either directly on GABA-A receptors, or through ERβ, resulting in a more dramatic change in GAD67-ir levels than expected based on the effects of flutamide in blocking actions at androgen receptors. However, the relative involvement of GABA-A receptor activation or ERβ in the effects of DHT would depend on the availability, and concentration, of the appropriate enzymes in these brain regions, and could contribute to

the differences in the effects of DHT in the BLA and CEA found in this study, or alternatively, as discussed previously, effects may emerge on a different developmental timeline in each subregion. One recent study has demonstrated significant brain region- and age-dependent changes in *de novo* synthesis of androgens and estrogens in the brain, as well as region-dependent expression of the enzyme, aromatase, which likely plays an important, but often unacknowledged, role in normal sexual differentiation in the brain (Konkle and Mccarthy, 2011). Although the levels of other steroidogenesis enzymes were not characterized in the present study, it is possible that variations in the concentration and time of expression of these enzymes could contribute to the relative importance of GABA-A and ERβ receptor activation by DHT in these different brain regions.

The lack of a sex difference, or effects of exogenous androgens, on GAD67-ir in the mPFC are somewhat surprising, given existing research on the importance of androgens in organizing other aspects of the mPFC (Pallarés et al., 2014; Mogi et al., 2015), including our own findings of prenatal androgens altering levels of DNMT3a-ir in the mPFC (Green and Perrot, unpublished data, see Chapter 4), and prenatal stress altering GAD67-ir levels in a sexually dimorphic pattern at this age (Green and Perrot, unpublished data, see Chapter 5). However, some existing studies point to an important role of prenatal androgens (Pallarés et al., 2014), as well as activation of estrogen receptors (Westberry and Wilson, 2012), in shaping PFC development. Thus, it is possible that postnatal androgens are less important than prenatal androgens, or postnatal estrogens, in shaping GABAergic development in this region. But, we also did not find a sex difference in vehicle treated males and females at this time, which could indicate that PND15 is not an appropriate time point to capture underlying sex differences in GAD67-

ir in this region, and a more complete developmental time course of GAD67-ir changes is required to investigate this further. Supporting this idea, a recent study in male mice reported that prenatal exposure to an immune challenge resulted in changes in PFC GAD65/67 protein levels that were found in adult, but not peripubertal male mice (Richetto et al., 2014), while another has reported that changes in parvalbumin levels resulting from early life stress emerged at later juvenile (female) and adolescent (male) time points in the PFC (Holland et al., 2014).

6.4.1. Concluding Remarks

Taken together, these results suggest that changes in the GABAergic system during the postnatal period are occurring in some brain regions associated with anxiety/depressive behaviours, as well as stress responding. Both endogenous sex differences, and a partial effect of androgens, were observed in the PVN and amygdala at our PND15 time point. Importantly, though this work points to a role of androgens in GAD67-ir cell density at this age, there is still much work to be done to characterize the development of sex differences in these brain regions, and this serves mostly to highlight the lack of existing research on sex differences in developmental GABA outside of reproductive and seizure behaviours. Future studies are needed, including a detailed ontogeny of GABAergic development across early life, as well as into juvenile, adolescent and adult time periods. Further, a more complete understanding requires investigation of other aspects of the GABAergic system, such as changes to receptor subunits, chloride co-transporter ratios, or other markers of GABA activity. Despite this, our work suggests that hormonally driven changes in GABA during the postnatal period

could provide one important mechanism linking sex differences, early life stress, and the role of the amygdala in vulnerability to anxiety and depressive disorders.

TABLE 6.1. SUMMARY OF THE EFFECTS OF ANDROGENS ON LIMBIC BRAIN REGIONS IN WHICH GAD67-IMMUNOREACTIVITY DENSITY WAS QUANTIFIED.

Brain Region	Sex Difference?	+DHT in Females	+Flutamide in Males
PFC	M = F	≠ F	≠ M
NAC-C	M = F	≠ F	≠ M
NAC-Sh	M = F	≠ F	≠ M
PVN	M < F	≠ F	≠ M
BLA	M > F	↑ F	< > M
CEA	M > F	↑ F	< > M

Summary of significant sex and treatment effects on offspring density of DNMT3a-ir by sex, or treatment with dihydrotestosterone (DHT) or the anti-androgen flutamide.. ↑ or ↓ indicates direction of effects, and ≠ indicates no effects of treatment. M = male, F = female. PFC = prefrontal cortex NAC-C = nucleus accumbens, core, NAC-Sh = nucleus accumbens, shell, PVN = paraventricular nucleus of the hypothalamus, BLA = basolateral amygdala, CEA = central amygdala.

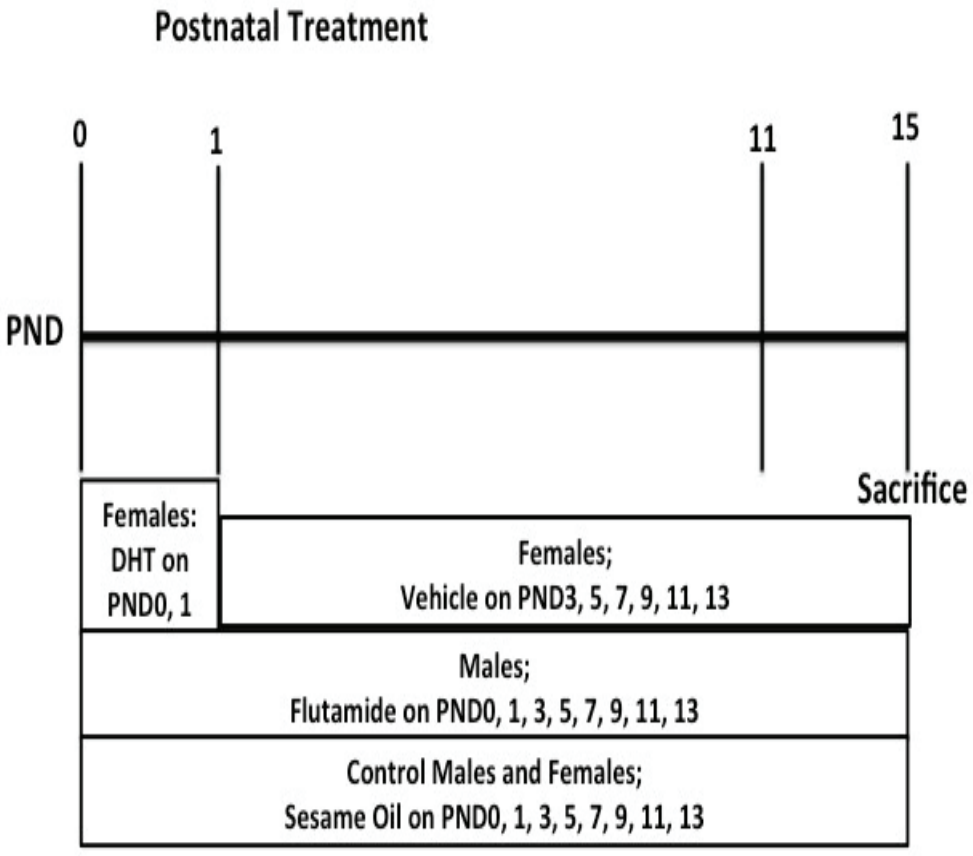

FIGURE 6.1. TIMELINE OF POSTNATAL HORMONE INJECTIONS.
Starting on the day of birth, postnatal day (PND) 0, female offspring were randomly assigned to receive subcutaneous dihyrotestosterone (DHT; 100µg/50µl) on PND0 and PN1, then vehicle control (50µl; 5% ethanol in sunflower oil) on PND3, 5, 7, 9, 11, 13, or vehicle control throughout. Male offspring were randomly assigned to receive subcutaneous flutamide (50 mg/kg in 50ul) or vehicle control on PND0, 1, 3, 5, 7, 9, 11, 13. All pups were sacrificed on PND15.

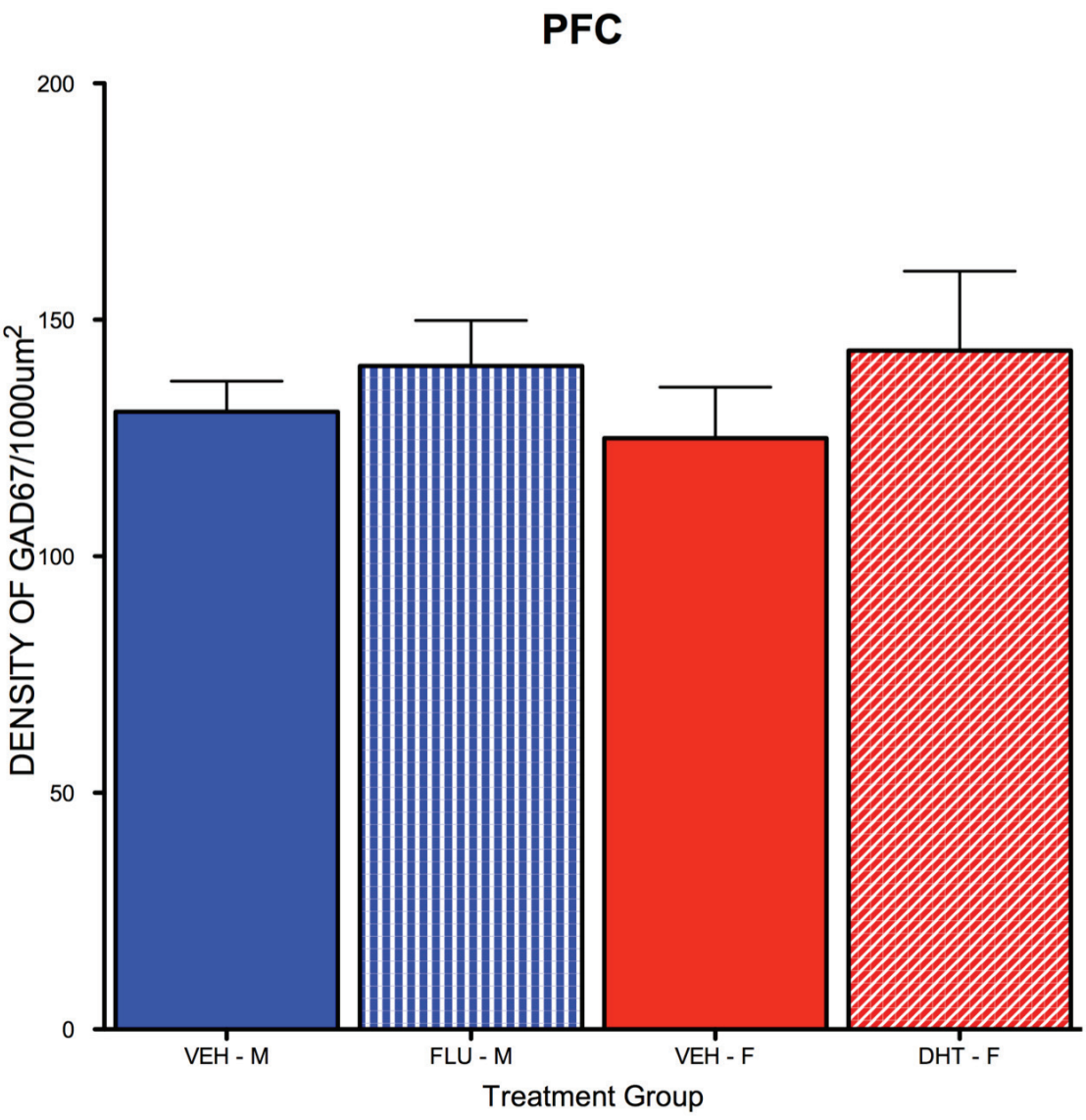

FIGURE 6.2. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE MEDIAL PREFRONTAL CORTEX ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of GAD67-ir cells in the mPFC of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 6.1. Posthoc comparisons revealed no significant differences.

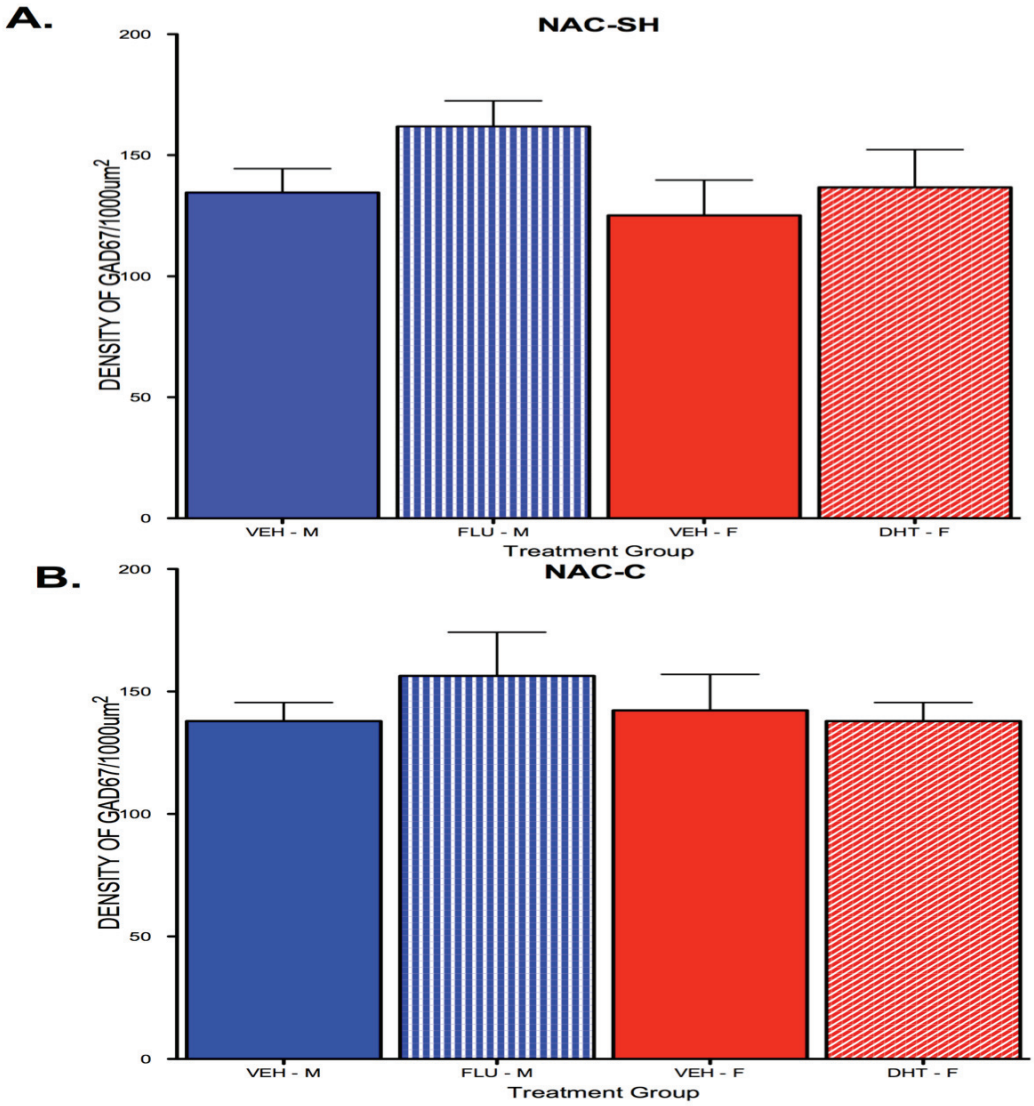

FIGURE 6.3. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE NUCLEUS ACCUMBENS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of GAD67-ir cells in the **A.** Nucleus Accumbens – Shell (NAc-Sh) and **B.** Nucleus Accumbens – Core (NAc-C) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 6.1. No significant group differences were found.

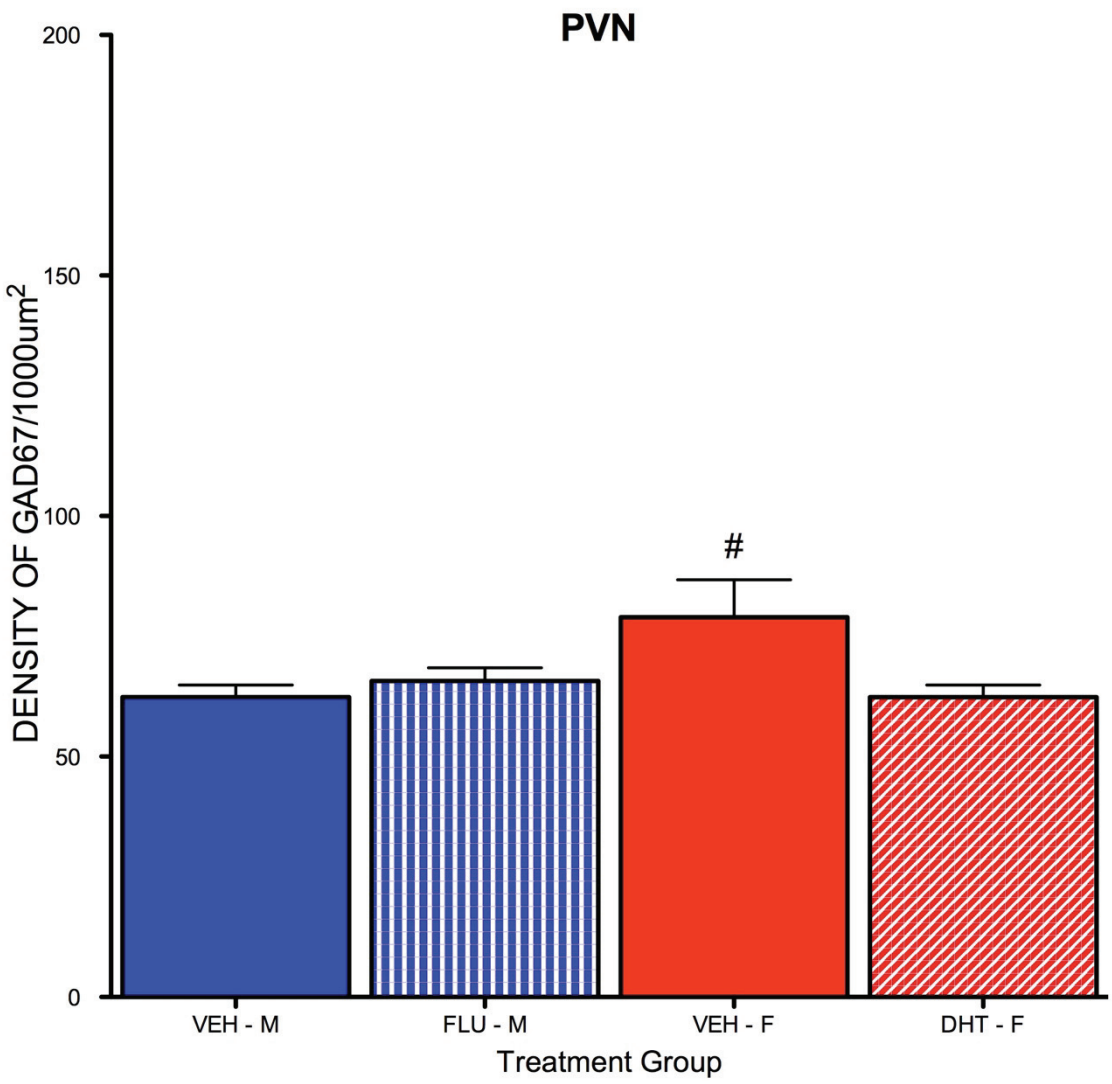

FIGURE 6.4. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of GAD67-ir cells in the Paraventricular Nucleus of the Hypothalamus (PVN) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 6.1. Posthoc comparisons revealed # a trend towards a difference from male vehice controls, $p < 0.055$.

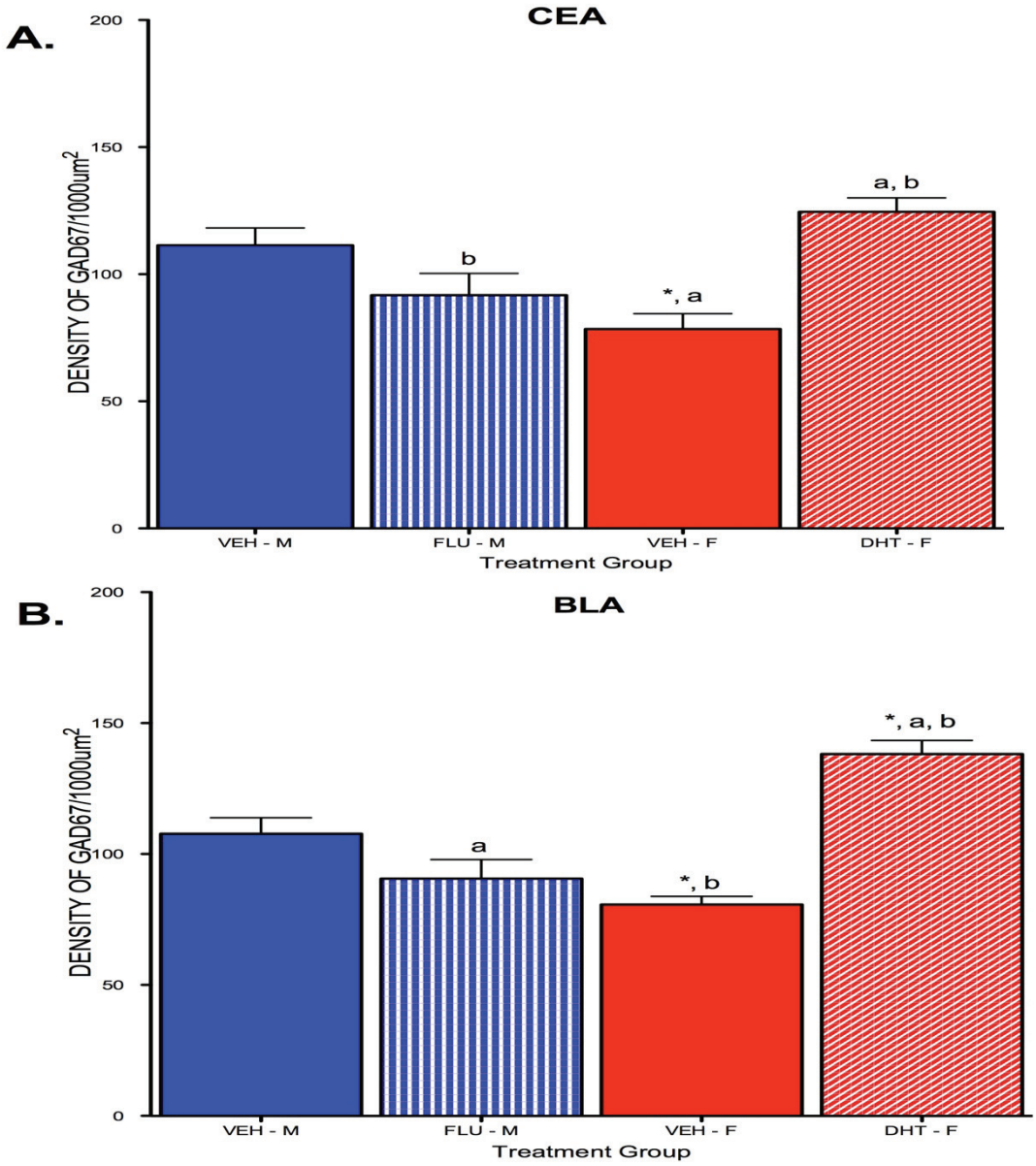

FIGURE 6.5. DENSITY OF GAD67-IMMUNOREACTIVE CELLS IN THE AMYGDALA ON PND15 IN MALE AND FEMALE OFFSPRING AFTER VEHICLE OR ANDROGEN TREATMENTS.

Density of GAD67-ir cells in the **A.** Centreal Nucleus of the Amygdala (CeA) and **B.** Basolateral Nucleus of the Amygdala (BLA) of postnatal day (PND) 15 male (M) and female (F) offspring that received subcutaneous injections of vehicle control (VEH), the anti-androgen flutamide (FLU), or dihydrotestosterone (DHT) according to the protocol described in Figure 6.1. No significant group differences were found. Posthoc comparisons revealed * significantly different from vehice control males. Bars denoted by the same letters are significantly different from each other, all $p < 0.05$.

CHAPTER 7 - CONCLUSION

7.1. – Integration of Findings, Limitations, and Future Directions

In this thesis, I have examined the importance of both sex differences (and underlying gonadal hormones) and the early environment (prenatal predator stress – PPS, and/or early enhanced housing – EHC) on both brain and behavioural changes at an early juvenile time point, using markers of either epigenetic changes (DNMT3a) or GABAergic changes (GAD67), and related some of these changes to observed anxiety and depressive behaviours across a developmental trajectory.

A major contribution of this work is in the behavioural testing of animals at multiple time points. Though the literature linking models of prenatal stress to adult anxiety/depressive behaviours is well established, it is only recently that researchers have begun to focus on early life, and, as reviewed in Chapter 1, there are still important gaps in our understanding of time points before puberty. Further, very few studies have examined changes across developmental time in the same animals, and those that exist often fail to include females, despite the clinical importance of sex differences in neuropsychiatric disorders and the parallel timing of sexual differentiation in the brain with early life stress paradigms. A detailed discussion of the behavioural results is present in Chapter 2, but overall, we found that many of the group effects of prenatal stress reported in the literature did not emerge until adult testing. Adult males demonstrated increased OFT anxiety and altered FST active behaviours, as well as reduced rough and tumble social play during the peripubertal period, which was partially rescued by EHC, while adult females exhibited sucrose anhedonia, which was prevented by EHC. These

results highlight the importance of including both sexes in models of prenatal stress, and, as discussed in Chapter 2, suggest that our novel, ethological stressor may be an interesting new paradigm for more 'psychological' studies of anxiety and depressive behaviours in animal models.

Of particular interest to our developmental focus is that behaviours at the juvenile time point did not correlate directly with adult behaviours in the same task – for example, immobility in the juvenile forced swim test did not predict immobility in the adult forced swim test. However, some juvenile behaviours (rearing in the OFT and FST immobility) were good predictors of other adult anxious (male OFT) and depressive (female sucrose preference) behaviours, respectively. This research highlights several intriguing possibilities – first, that these particular behavioural tasks (center time in the OFT and immobility in the FST) do not accurately model anxiety/depressive behaviours in juveniles. As described in Chapter 1 and Chapter 2, the open field relies on a conflict between desire for exploration and fear of open spaces (for review, see Andrews et al., 2014), and, as discussed in Chapter 2, it is possible that behaviours considered 'anxious' in adult animals may be more adaptive at this early age, when strategies for avoiding predators and/or acquiring food may be very different, while behaviours such as rearing, which are often considered more reflective of activity/locomotion, could (as suggested in our results) better reflect vigilance or anxiety in juveniles. Similarly, many researchers question the validity of the forced swim test in modeling behavioural 'despair' in adult animals (reviewed in Molendijk and de Kloet, 2015), arguing that it instead is an adaptive learned response to conserve energy in the face of an inescapable situation, and this may also be the case in juveniles.

Alternatively, the more 'clinical' anxiety/depressive symptoms being modeled by these tasks may not emerge until later life, and these other behaviours (rearing, immobility) reflect underlying neural changes that will ultimately manifest as anxiety/depressive behaviours. Given that the majority of clinically diagnosed cases of depression emerge after adolescence (Kessler et al., 2001), this is an interesting potential clinical parallel. In order to investigate these possible explanations, future studies should examine a broader battery of anxiety/depressive tests in pups at early juvenile time points, including for example, other anxiety tasks such as the elevated plus maze, conditioned fear in response to a predator, etc. and other 'depressive' tasks including for example, tail suspension, intracranial self-stimulation, or learned helplessness paradigms (reviewed in Czéh et al., 2016). Performing these test batteries at multiple ages, including time points through adolescence and adulthood, will help elucidate when/how these behavioural patterns first emerge, and how these behaviours might correlate across different tasks. Determining precisely when these behavioural changes first emerge may be particularly important to understanding the developmental trajectory of these disorders, and allow future research to focus on critical periods of vulnerability or intervention in the brain. Indeed, though I documented changes in adult animals that had not emerged in juveniles, we do not know exactly when they first arose other than after PND17 (during or after puberty, for example), and this is an important limitation when extrapolating to underlying brain and behaviour changes.

Another important result emerging from this work was that our enhanced housing condition (EHC) did not have many independent, long-lasting effects on behaviour, and some changes that were present in juveniles are no longer visible in adults. As discussed

in Chapter 2, effects of EHC may fade over time (Rosenfeld and Weller, 2012). However, EHC did act to prevent some of the effects of PPS in adulthood, which lends support to the hypothesis that EHC is a mild 'inoculation' stressor that may have effects primarily in mediating the response to later adult stressors (Cymerblit-Sabba et al., 2013; Connors et al., 2015) as discussed in Chapter 2. Further research is required to determine if a longer exposure period, or exposure during a different time period might have greater effects. As well, investigating whether our early life EHC paradigm is protective in the face of further adult stressors could help clarify this relationship. In either case, it is important to keep this in mind when interpreting the results of EHC on the brain at PND15, as changes at PND15 might not reflect long-term effects of EHC in the brain, and further studies of our neural markers at other developmental time points is required to investigate this possibility.

Despite these limitations, the research in Chapter 2 demonstrated that some sex-specific changes in behaviour were already present in the PND15 animals, and that these changes could predict some aspects of adult behaviour. This led me to investigate the brain at this age, looking for sex- and age-specific changes in potential neural mechanisms that could be contributing to these behaviours, as discussed in Chapters 3 (DNMT3a) and Chapter 5 (GAD67). Given that sex differences in these behaviours were already present by PND15, and results in Chapters 3 and 5 suggested sex differences in the brains at this age as well, I examined the importance of organizational gonadal hormones (androgens) in normal sexual differentiation in these brain regions/markers at this same time point, as described in Chapters 4 (DNMT3a) and 6 (GAD67). The results

of these experiments are discussed in detail in the corresponding chapters, as are their potential links to the behavioural changes found in Chapter 2.

Briefly, PPS increased DNMT3a-ir density in both males and females at this age, though there were sex differences in which regions were affected, with females showing increases in more regions than males, including the mPFC and NAc-Sh. Whether EHC could 'rescue' these changes was highly dependent on brain region and sex as well, and, as discussed in Chapter 3, some of these changes may correspond to the behavioural effects observed in Chapter 2. In Chapter 3, we observed sex differences in DNMT3a in some, but not all, of the brain regions examined (the PFC, BLA and PVN), and androgens partially mediated these differences in the amygdala and PVN, but not the PFC. This suggests a likely role for both androgens and estrogens in normal sexual differentiation of DNMT3a-ir in these brain regions. Taken together, these chapters point to a novel role for DNMT3a-ir in the sexually dimorphic development of brain regions associated with stress responding, as well as anxiety and depression, and suggests that organizational hormones may be involved in some of these changes. This research is particularly interesting as the majority of studies have either focused on adult changes in DNMT3a-ir in relation to these anxiety/depressive disorders, or a role of DNMT3a in establishing normal sexually dimorphic reproductive behaviours, and this is one of the first studies to highlight an early life intersection of these fields, opening up several exciting avenues of future research focus. For example, other markers of epigenetic changes, including other DNMTs, HDACs, etc., also need to be examined, as well as investigating which downstream neurotransmitter systems are being targeted by the changes in methylation observed.

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Unlike DNMT3a, the effects of PPS on GAD67-ir were more variable in their direction, and highly dependent on sex, with PPS decreasing GAD67 in males, and increasing it in females, with effects varying between brain regions. EHC prevented/rescued many of these changes, but also independently altered GAD67-ir in several regions in females, and some, but not all, of these changes could be related to our observed anxiety/depressive behaviours, as discussed in Chapter 5. Unlike DNMT3a-ir, baseline sex differences in GAD67-ir were primarily found in the amygdala, with a more complex role for organizational androgens in establishing these differences. As with the research on DNMT3a-ir, these are some of the first studies to document sex-specific changes in in GAD67 in a model linked to anxiety/depressive disorders prior to adolescence, as well as to suggest GAD67 may be involved in normal sexual differentiation in the early development of the amygdala via organizational androgens. This offers an intriguing new avenue for potential future research into the developmental origins of anxiety and depression, as well as possible new directions for research into interventions and treatments. There is however, still much work to be done to better understand these changes, including a more complete characterization of other aspects of the GABAergic system, such as neuronal subtypes, receptor subunit composition, etc.

Notably, one of the appealing aspects of GABA as a neurotransmitter system involved in mediating our behavioural changes was previous research linking changes in GAD to epigenetic changes in DNMT3a, as discussed in Chapter 5 (reviewed in Menke and Binder, 2014). Overall, changes in DNMT3a and changes in GAD67 in our model did not appear to associate very strongly, often occurring in different directions, or in

different brain regions in response to the various environmental interventions or hormonal treatments. It is possible then that DNMT3a-ir is not involved in mediating the changes in GAD67 in our model, and they are independently altering aspects of early development. For example, changes in density of GAD67-ir could be driven by other epigenetic mechanisms, such as changes in HDAC or DNMT1. Alternatively, transient changes in DNMT3a could be preceding those seen in GAD67, altering methylation patterns in GAD1 promoters that would be reflected in a later time point. As discussed in Chapter 3, overall levels of DNMT3a-ir do not provide information about which genes in particular are being methylated, and it is possible that while DNMT3a-ir is methylating GAD1, it is also altering a myriad of other genes; resulting in the differing patterns of GAD67 and DNMT3a-ir we are seeing overall. Additional studies are required to investigate these possibilities and to parse out the relationship between GAD and DNMT3a in this model. Further, in the case of our studies on both GAD67 and DNMT3a, it is important to note that this research is based on immunohistochemical analysis of the density of cells, which is not the same information as the total amount of protein or mRNA in the region, nor a direct representation of how these proteins are operating to change electrophysiological signaling properties in these regions. While this research provides important information about anatomical localization, and the number of cells expressing these markers, research using other techniques including western blotting, qPCR, and electrophysiology would greatly expand our understanding of how these markers are altering the neurophysiology of these brain regions.

The primary limitation of our neuroanatomical studies on both GABA and DNMT3a is that, although we chose our particular time point based on behavioural

changes in Chapter 2, as well as the existing literature suggesting PND15 would be immediately following the end of the normal period of sexual differentiation, ultimately, it is still only one time point. As has been illustrated by our review of the literature, many of the changes in the adult brain/behaviour are likely established across early developmental time, and without a more systematic characterization of the 'normal' and abnormal developmental trajectory of these brain regions, it is impossible to fully understand the process of differentiation, and the critical periods that are most vulnerable to environmental influences, particularly in case of transient changes at earlier or later ages, that may be overlooked by the focus around PND15. Our work provides a valuable jumping off point - highlighting brain regions and epigenetic/neurotransmitter systems that are likely important in early life, but an important future direction of research will be to apply this developmental trajectory approach to these regions and neural markers, across several early prenatal/postnatal time points, as well as through adolescent and adult development. Further, even a full anatomical and functional characterization of the development of these brain regions can only be correlated with changes in behaviour, and eventually, researchers will need to directly disrupt the development of these systems at different developmental time points and observe whether the anxiety/depressive phenotypes are recreated.

Despite these limitations, this research provides important evidence for our novel ethologically relevant prenatal predator stress as a risk factor in animal models of anxiety/depressive disorders, with several interesting changes to behaviour in early life that make this an attractive model for future studies modelling childhood risk factors. Both GABAergic and epigenetic changes appear to play a role in the developmental

trajectory of these changes, as do sex differences, and the process of early sexual differentiation. By beginning to understand the consequences of both sexual differentiation, and early life stress/enrichment in these brain regions, the hope is to provide new hypotheses for research into early identification of deficits that underlie these neuropsychiatric disorders, as well as new potential avenues for treatment.

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