EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON INTERACTIONS BETWEEN STRESS AND DOPAMINE SYSTEMS: A POTENTIAL PATHWAY TO INCREASED VULNERABILITY TO SUBSTANCE USE DISORDERS

by

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ABSTRACT

Effects of prenatal alcohol exposure (PAE) on central nervous system function include an increased prevalence of substance use disorders (SUDs). Dopaminergic systems provide a key neurobiological substrate for SUDs. The hypothalamic-pituitary-adrenal (HPA) axis and dopamine systems have overlapping neurocircuitries, with stress altering dopamine pathways implicated in drug-related reinforcement and motivation, and conversely drug exposure activating stress systems, enhancing sensitivity to subsequent stressors. PAE alters both HPA and dopaminergic regulation, resulting in increased HPA tone and an overall reduction in tonic dopamine activity. Thus, alterations in HPA-dopamine interactions in PAE subjects may provide a neurobiological mechanism underlying enhanced vulnerability to SUDs. Adult Sprague-Dawley offspring from PAE, pair-fed, and *ad libitum*-fed control groups were examined. In Chapter 2, the effects of PAE and stress on basal regulation of stress and dopamine systems are discussed. Subjects were subjected to either chronic variable stress (CVS) or no stress conditions, and corticotropin releasing hormone (CRH) mRNA, as well as glucocorticoid and dopamine receptor expression, were measured under basal conditions. In the hippocampus, glucocorticoid receptor (MR) mRNA levels were lower in PAE than control females under non-CVS conditions, while CVS resulted in broader upregulation of MR in PAE compared to control males. A decrease in dopamine receptors was observed following CVS exposure in control but not in PAE subjects. Overall, PAE enhanced sensitivity to CVS and attenuated the effects of chronic stress on basal dopamine receptor expression, and did so in a sexually-dimorphic manner. In Chapter 3, repeated exposure to *d*-amphetamine (AMPH) induced behavioral sensitization in PAE but not control subjects, and this behavioral measure is positively correlated with vulnerability to SUDs. The current study also assessed cross-sensitization between AMPH and stress, and indeed PAE facilitated cross-sensitization between AMPH and stress, and did so in a sexually dimorphic manner. PAE altered AMPH-stress interactions, and did so in a manner consistent with increased neurobiological vulnerability to SUDs. Together, the present results enhance our understanding of PAE effects on the cross-talk between dopamine and stress systems, and provide insight into underlying mechanisms influencing the increased prevalence of SUDs among individuals with an FASD.

PREFACE

The identification and design of the research included in the present dissertation was primarily created by Kristina Uban, under the direct supervision of Dr. Liisa Galea and Dr. Joanne Weinberg along with valuable feedback from Galea and Weinberg laboratory members during regular meetings. Kristina Uban was the primary executer of all experiments. All data analysis within the dissertation, as well as preparation of the manuscripts, introduction (chapter 1) and discussion (chapter 4) was performed by Kristina Uban under direct supervision by Dr. Liisa Galea and Dr. Joanne Weinberg. Dr. Wendy Comeau also provided feedback on the introduction and discussion of the present dissertation.

Chapter 2: Assistance with the research was received by the following individuals with the following tasks: Dr. Wendy Comeau, Linda Ellis, Wayne Yu, Tamara Bodnar, and Welan Dionela assisted with the breeding and execution of animal work; Wayne Yu assisted with RIAs; Linda Ellis assisted with IHC and ISH; Dr. Wendy Comeau assisted with brain slicing; Stephanie Leiblich, Caitlin Bauermeister, Welan Dionela, and Farinaz Poursoltani assisted with tissue mounting; Leanne Chew and Andrew Choe assisted with scanning and measuring MR and GR; and no assistance was received with imaging of CRH or DA-Rs.

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DEDICATION

This dissertation is dedicated to all individuals with an FASD and their loved ones. The value of the present work is determined by the benefit it brings to those affected by FASD.

"Nothing in life is to be feared. It is only to be understood". -Marie Curi

Chapter 1: Introduction

1.1 Overview

The relationship between stress and dopamine system interactions has received increased interest for its role in vulnerability to, or resilience against, substance use disorders. Importantly, an increased prevalence of substance use disorders is observed among individuals with a fetal alcohol spectrum disorder (FASD). However, the effects of prenatal alcohol exposure (PAE) on alterations between stress and dopamine systems remain an understudied area of research. The present dissertation aims to fill this gap. Mechanisms that may influence mental health problems in individuals with FASD include underlying dysregulation of stress and dopamine systems. Thus, the aim of this dissertation is to elucidate alterations in the interaction between central stress and dopamine systems. The present introduction will highlight issues directly relevant to mechanisms underlying fetal origins of adult disorders, the impact of stress on mental health, and increased vulnerability to substance use disorders. Pre-clinical and clinical evidence of alterations observed among individuals with FASD, as well as animal models of PAE, will be integrated into these theories. The aims of the dissertation include: 1) connecting evidence from past literature on stress system alterations with dopamine system alterations following PAE; 2) elucidating the bi-directional interaction between stress and dopamine systems following PAE; and 3) elucidating sexually-dimorphic effects of PAE on stress and dopamine system interactions, as well as correlating alterations in gonadal and stress hormones.

1.2 Importance of early life environment

Plasticity of the developing brain is subject to sensitive periods, when environmental conditions significantly impact developing brain regions. Several developmental periods are associated with increased levels of neuroplasticity. These periods range from the prenatal period to early adulthood, with the early stages of central nervous system (CNS) development during the prenatal period being especially plastic. The prenatal-perinatal period is the time during which the majority of CNS development occurs and therefore signifies a vulnerable period during the lifespan for perturbations. Specific brain regions have different periods of enhanced sensitivity across the lifespan; therefore the relationship between brain development and early life exposure to adversity is highly dependent not only on the type of insult but also on timing of the insult. Interestingly, the effects of early life adversity on brain development present at varying times, with some regions (e.g. prefrontal cortex) showing alterations soon afterward, whereas other regions (e.g. hippocampus) exhibit a delay in the presentation of these alterations (Andersen and Teicher, 2009). The differences in 'incubation' lengths of early life adversity effects likely depends on the rate of maturation of a brain region, with some regions developing more slowly over time (e.g. prefrontal cortex), while other regions (e.g. hippocampus) experience discrete periods of rapid maturation. For example, an increase in hippocampal gray matter can be observed in children throughout adolescence and into early adulthood (Gogtay et al., 2006); however, childhood abuse produced the greatest reductions in hippocampal volume if the abuse occurred between the ages of 3 and 5 years or between 11 and 13 years (Andersen et al., 2008). This suggests discrete periods of enhanced susceptibility of the hippocampus to early life adversity, and similarly, these periods may be highly responsive to interventions.

Fetal origins of adult disease: The link between prenatal environment and later physical and mental health in adulthood has long been recognized (Seckl and Holmes, 2007), specifically as it pertains to adverse environments including poor nutrition, exposure to teratogenic substances such as alcohol or nicotine, and maternal stress. Thus, it is reasonable to propose that several adult diseases may have common developmental origins, potentially as a consequence of fetal programming. *Fetal programming* refers to the concept that adverse prenatal environments can program developing and/or maturing neurophysiological systems, resulting in altered sensitivity of these systems throughout life. Stress systems are particularly vulnerable to programming by early life events and may provide a final common pathway through which many different early life events can alter long-term outcomes (Meaney and Szyf, 2005, Seckl and Holmes, 2007, Weaver et al., 2004, Welberg and Seckl, 2001, Young, 2002).

Perhaps the most famous example of fetal programming in humans resulted from the Dutch famine from 1944-1945. The majority of pregnant women in this geographical location were significantly undernourished for either the beginning of pregnancy, or throughout the entire pregnancy. The resulting children from this time period appeared relatively healthy, but then suffered from a number of diseases in adulthood, such as cardiovascular problems, impairments in glucose tolerance, and obesity (Roseboom et al., 2001). Interestingly, the effects of *in utero* malnutrition on adult disease varied depending on timing of exposure (Roseboom et al., 2001). Resulting from a variety of maternal conditions, birth weights have since then been shown to serve as a rough marker of vulnerability to adult disease, with birth weights within lower and higher ends of the distribution indicating enhanced risk in adulthood (Barker, 2001, Godfrey and Barker, 2001). Importantly, enduring alterations in stress reactivity have a significant impact on

brain development (reviewed in (Andersen and Teicher, 2009), with long-term dysregulation of stress systems producing physiological, neurobiological and behavioral problems, which may pave the road for vulnerability to later psychiatric conditions.

Along similar lines, the *diathesis-stress* model hypothesizes that a genetic and/or biological condition (diathesis) predisposes an individual to later adverse environmental and/or life events (stressors), resulting in problematic behavioral, physiological and/or psychiatric conditions. It has also been hypothesized that stressors are additive over one's life span. Both individual and sex differences are observed at the tipping point at which an individual begins to display a given disorder (Becker and Hu, 2008). The adaptive processes responsible for maintaining homeostasis in response to stressors is referred to as *allostasis* (McEwen, 2002), and the impact of the accumulation of life stress is referred to as *allostatic load* (Juster et al., 2010, Lupien et al., 2009, McEwen, 2007) (described further in section *1.5*). Individuals with severe underlying vulnerabilities may develop diseases or disorders following only a small amount of stress. Such individuals would then be protected from the development of disorders only in the context of enhanced protective factors. In order to understand the etiology of adult disorders, it is essential to understand the diathesis, stress, as well as potential protective factors. The present dissertation focuses on the impact of the prenatal environment, specifically *in utero* alcohol exposure, on developing neural systems, which may result in life-long neurobiological alterations and problematic physiological or behavioral responses in later life.

1.3 Fetal alcohol spectrum disorder

One serious consequence of repeated alcohol consumption during pregnancy is the development of fetal alcohol spectrum disorder (FASD) in the children. The estimated prevalence of FASD is around 9 cases per 1,000 births in Canada and the USA (Sampson et al., 1997, Thanh and Jonsson, 2010), which is believed to be a conservative estimate. The dose and duration of alcohol exposure a fetus will experience is affected by the interaction between the rate of metabolism and the pattern of alcohol consumption, with higher maternal blood alcohol levels (BALs) having a greater impact on fetal development. The long-lasting alcohol-related effects of FASD can include; 1) growth deficiencies, 2) physical abnormalities, and/or 3) damage to the central nervous system (CNS) (Calhoun and Warren, 2007). A diagnosis of Fetal Alcohol Syndrome (FAS), which is the most severe outcome, requires all three alcohol-related effects to be present. Children exposed to lower doses of alcohol, who show only some of the facial features and neurobehavioral alterations can receive a diagnosis of partial FAS, while those having no facial dysmorphology yet showing neurobehavioral alterations may be diagnosed with Alcohol-Related Neurodevelopmental Disorder (ARND) (Stratton et al., 1996). Although not a clinical term or diagnosis, FASD is used as an 'umbrella' term to encompass all individuals affected by *in utero* alcohol exposure and the wide range of deficits or abnormalities that may ensue from the exposure. Damage to the CNS following alcohol exposure *in utero* can include neurocognitive impairment (cognitive function, executive function, learning and memory), impairment in self-regulation (attention, hyperactivity, impulsivity, behavioral regulation), and deficits in adaptive functioning (communication, social behavior, motor deficits) (Rasmussen, 2005, Spohr et al., 2007, Streissguth and O'Malley, 2000). Furthermore, whereas facial

dysmorphologies and growth deficiencies may become less prominent with time, perturbations to the CNS persist into adulthood and can produce relatively permanent alterations in behaviour (O'Connor and Paley, 2009, Rasmussen, 2005, Spohr et al., 2007). Thus, FASD has been termed the 'invisible disability'.

The wide range of effects that occur following PAE is likely related to variations in the amount of alcohol, and the timing and duration (i.e., stage of embryonic development) of exposure. For example, metabolism of alcohol by the placenta can reduce the alcohol levels in the blood reaching the fetus to some extent, and rates of metabolism are known to vary in the human population. Additionally, acetaldehyde is a metabolite produced from the breakdown of alcohol, and this metabolite is a probable carcinogen that may in itself have harmful effects on the developing fetus (Karl et al., 1988). Even more, maternal alcohol consumption has been shown to impair placental transport of key nutrients such as amino acids, biotin, vitamin B_6 and fatty acids (Fisher et al., 1982, Fisher et al., 1981), which can indirectly affect fetal growth and development. High levels of exposure during the first 8 weeks of pregnancy (embryonic stage), are more likely to cause structural deformities. In contrast, even relatively low levels of exposure during synaptogenesis and further refining of CNS development occurring in the third trimester can cause life-long abnormalities of CNS connectivity and functioning. Although perhaps less severe in nature, even moderate changes in connectivity and functioning during development may have profound effects on the maturation and function of the CNS throughout life. In addition, it has been shown that young adults with FASD have similar neuropsychological deficits or secondary disabilities regardless of their diagnosis of FAS, partial FAS or ARND (O'Connor and Paley, 2009, Pei et al., 2011, Rasmussen, 2005, Streissguth et al., 2004). 'Secondary' disabilities refer to deficits resulting from primary deficits, including mental

health problems. However, mental health problems may have a primary basis as well, as pre-existing alterations in neurobiology likely influence an individual's vulnerability to developing mental health problems including substance use disorders.

Importantly, alcohol consumption alters maternal stress systems. Maternal stress hormones can pass through the placental barrier, and alter the developing fetal stress systems. Additionally, alcohol itself is lipid soluble, and readily passes through the placental barrier, and acts on the developing fetal stress systems (Idanpaan-Heikkila et al., 1972). While aldehyde dehydrogenase activity in the placenta allows it to metabolize low levels of alcohol, high levels of alcohol may have major adverse effects on the fetus (Burd et al., 2007, Meier-Tackmann et al., 1985). The mother and fetus form an interrelated functional unit, therefore alcohol-induced changes in the mother have implications for fetal development. Of particular relevance for the present research, alcohol-induced maternal-fetal endocrine imbalances could possibly contribute indirectly to the etiology of FASD (Anderson, 1981). Alcohol readily crosses the placenta; thus, directly affecting developing fetal tissues, including those involved in endocrine function. In addition, alcohol-induced changes in endocrine function can disrupt the hormonal interactions between the maternal and fetal systems; thus affecting the development of fetal metabolic, physiological and endocrine functions. For example, maternal corticosterone crosses the placenta, suppressing endogenous activity of fetal stress systems, whereas alcohol crossing from the mother to the fetus through the placenta has the opposite effect, activating fetal stress systems (reviewed in (Zhang et al., 2005)). Interestingly, one study found that maternal alcohol consumption increased placental weights compared to those in control dams, but decreased essential amino acid concentrations in the alcohol exposed fetus without altering the capacity of the placenta to take up amino acids (Gordon et al., 1985). Maternal alcohol consumption alters

the transfer of nutrients from the placenta to the fetus. This effect is likely confounded by the presence of alcohol itself interfering with the fetus's ability to utilize nutrients that do pass through the placenta (Weinberg, 1984).

Overall, re-programming of the developing fetal stress systems, as a result of both indirect effects of maternal stress hormones and direct effects of alcohol itself, result in life-long dysregulation of these stress systems (Hellemans et al., 2010a). The timing and dose of alcohol exposure has a significant impact on the observed results, and in humans, the timing and dose of alcohol consumption often varies to a great degree among mothers. In the current dissertation a rat model of FASD was utilized that targets disruption during the human equivalent of the first and second trimesters. This model is described in more detail in section *1.4*. Importantly, stress systems goes through a significant degree of development during the first to second trimesters and is highly susceptible to neonatal experience (Plotsky et al., 1993). Importantly, the alterations in stress-regulation are specific to the type of early life stressor and timing of exposure (Andersen and Navalta, 2004, Andersen and Teicher, 2008); therefore resulting in a unique role in vulnerability to, or resilience against, substance use disorders.

1.4 Animal models of FASD

A main strength of utilizing animal models to study the effects of prenatal alcohol exposure is the ability to elucidate underlying mechanisms, as well as control variables that cannot be controlled in the human situation (i.e. dose, timing, nutrition, poly-drug use). The development of suitable animal models has been crucial in investigating the wide range of

potential effects of PAE, the factors that can influence the severity of effects observed, and the mechanisms mediating those effects. The investigation of FASD has utilized a range of animal models including, but not limited to, Caenorhabditis elegans (*C.elegans*) (Davis et al., 2008), Drosophila (McClure et al., 2011), Danio rerio (zebra fish) (Bilotta et al., 2002), as well as the beagle (Ellis and Pick, 1980), sheep (Lafond et al., 1985), and rodents such as the mouse (Lochry et al., 1982) and rat (Weinberg et al., 1986, West et al., 1984), each of which can model particular aspects of the disorder.

Non-mammalian and mammalian models of FASD: The smaller nervous systems of non-mammalian models have made them especially useful in elucidating teratogenic effects across generations, due to the relatively short reproduction and development timeline (Davis et al., 2008, Heaton et al., 1992). Moreover, these models have been particularly useful for studies on genetics (Gerlai, 2003, Su et al., 2001), providing insight into the role of cell signaling pathways in alcohol's adverse effects (Ahlgren and Bronner-Fraser, 1999, Morgan and Sedensky, 1995). However, it is likely that interactions between direct and indirect (maternally-mediated) effects of alcohol are responsible for its adverse effects in mammals (Randall et al., 1990). Alcohol is known to act on many different target molecules; therefore, multiple mechanisms, activated at different stages of development or at different dose thresholds of exposure, probably contribute to the diverse phenotypes seen in FASD (Goodlett et al., 2005). As well, alcohol could disrupt development through endocrine or neuroendocrine imbalance and altered maternal-fetal hormonal interactions (Hellemans et al., 2010a, Zhang et al., 2005), and allow for investigation of the role of placental transfer of alcohol itself, as well as alterations in nutrition, and maternal hormones.

Important considerations for the effects produced by PAE: In mammalian animal models of FASD, a number of factors need to be addressed and/or considered including: timing of alcohol exposure, blood alcohol levels (BALs), mode of alcohol administration, nutritional considerations, and maternal/pup interactions. For example, much of the variability in the effects of alcohol on the developing embryo-fetus may be attributed to the timing of exposure. This has been underscored in the research of Sulik and colleagues (Sulik and Johnston, 1983, Sulik and Schoenwolf, 1985), who demonstrated that the timing of exposure is crucial to the development of craniofacial anomalies in mice, a signature feature of FAS in humans. For example, alcohol exposure as early as gestational day seven reduced the size of the neural plate, which corresponded with facial dysmorphologies in the eye, philtral region of the upper lip, and the forebrain of fetal mice (Sulik and Johnston, 1983). Importantly, this period of mouse development corresponds with the human embryonic stage when many women are unaware of their pregnancy (Sulik and Johnston, 1983).

In humans, the development of FAS and other alcohol-related effects depends on alcohol exposure *in utero*. However, in many mammals, including the rat, offspring are born less developed than the human fetus, with pups being born at a point in development, and particularly brain development, that is roughly equivalent to the end of the second trimester in humans (Dobbing and Sands, 1979). The third trimester in humans is a critical developmental phase that includes the brain growth spurt (Dobbing and Sands, 1979) and the development of immunocompetency (Kimura et al., 1985). In rats this would encompass the postnatal period from birth to approximately postnatal day 15 (Dobbing and Sands, 1979). However, the placenta plays a significant role in mediating alcohol's teratogenic effects; therefore, post-natal alcohol

exposure to rodent pups cannot model the mediating role of the placenta, which produces additional confounds.

In humans, variations in genetics, age, weight, food consumption, metabolism, concomitant drug use and prior alcohol use all influence maternal BALs and therefore the level of alcohol to which the fetus will be exposed. The pattern of alcohol use, along with the timing of exposure, may be the best determinant of the potentially detrimental effects on the fetus, with the consensus being that higher BALs suggest potentially more severe effects (Bonthius and West, 1988, Chernoff, 1977, Pierce and West, 1986, Randall and Taylor, 1979). Rodents rarely self-administer a high enough volume of alcohol or a solution containing a high enough alcohol concentration to become intoxicated (with a few exceptions including animals bred to be "alcohol preferring"); therefore, making it difficult to achieve significantly elevated BALs during gestation.

It is important to consider whether dams will voluntarily consume a diet with a high enough alcohol content to elevate BALs sufficiently and subsequently produce FASD-like effects similar to those found in humans. In rodent models, various modes of administration have been utilized in FASD research, ranging from gavage or intubation, to intraperitoneal injections, alcohol vapor exposure (Kang et al., 2004, Lee et al., 1990), and self-administration methods including intravenous infusions, alcohol in the drinking water, and liquid diets (see (Lieber et al., 1989, Riley and Meyer, 1984) and (Ponnappa and Rubin, 2000) for a review). Forced alcohol administration using gavage/intubation, intraperitoneal injections and vapor chambers allow for excellent control over timing and dose, but often create additional stress for the subjects. Self-administration is generally the preferred method for chronic exposure, as it is minimally invasive, and more closely reflects the human situation producing greater face validity. The

construct validity of a voluntary alcohol consumption paradigm during gestation is better than models involving passive alcohol exposure throughout gestation. However, there are issues of construct validity not addressed by the current model of FASD used in the present dissertation. For example, in the human situation, women do not begin consuming alcohol at the start of gestation, but typically have been consuming alcohol for considerable periods of time prior to pregnancy, and respective male partners are typically also consuming alcohol. The fact that we begin alcohol exposure at the start of gestation is a limitation directly pertaining to construct validity of our paradigm. These limitations also come with strengths, however, as the present model allows for investigation of the effects of maternal alcohol exposure during the human equivalent of the first and second trimesters only. This produces greater understanding of the effects of timing on later neurobiology in the exposed offspring, as well as effects that can be attributed to the *in utero* environment.

Increased consumption of alcohol is concomitant with decreases in food consumption, in part owing to the increased calories derived from alcohol itself (Weinberg, 1984). Alcohol effects on food intake, nutrient absorption and utilization, and blood flow in the placenta can produce nutritional deficiencies among other effects in humans as well as animal models of FASD. For example, chronic alcohol consumption increases intake of "empty" calories (calories not linked to specific nutrients) while decreasing food intake and interfering with the availability and utilization of essential minerals and vitamins including vitamin A, zinc, folate, thiamine, choline, and B6 (for a more complete review see (Weinberg, 1984)). These nutrition-related issues produce confounds in all prenatal alcohol-related models. Although it is well recognized that an adequate control group that addresses these issues in their entirety does not exist, when possible it is prudent to include a pair-fed group that provides for some control related to the

effects of dietary restriction induced by alcohol consumption. Thus, a pair–fed group has been included in the present studies in additional to a standard control group to further control for some of the nutritional issues that occur in alcohol-consuming dams.

Pair-feeding in models of PAE: Most researchers in FASD studies use pair-fed (PF) or yoked controls to control for the reduced food intake that typically occurs with alcohol consumption. However, the issue of pair feeding is a complex one. Although pair-feeding is the standard procedure utilized to separate nutritional effects of alcohol from its direct effects, it serves as both an additional control group and as an experimental treatment group in itself (Glavas et al., 2007, Hofmann et al., 1999, Weinberg, 1984). For example, pair-feeding can only control for the reduced intake of the alcohol-consuming animals, but can never control for the effects of alcohol on absorption and utilization of nutrients. Indeed, alcohol intake always produces uncontrollable secondary nutritional effects that are part of its effects on the body. Secondly, pair-feeding is stressful to the dam impacting behavioural and physiological responses (Gallo and Weinberg, 1981, Hofmann et al., 1999, Weinberg and Gallo, 1982). In contrast to alcohol-consuming dams, who consume their diet *ad libitum*, PF dams receive a reduced ration of food, less than they would consume if given the same diet without alcohol and therefore consume their entire daily ration within a few hours of the food presentation. Despite the fact that experimental diets are formulated to provide optimal nutrition during pregnancy, underfeeding constitutes a mild prenatal stressor, which can itself influence offspring behavioral and physiological responses (Kwong et al., 2000, Levine and Wiener, 1976, Weinberg, 1989). Depending on the outcome measure, PAE and PF offspring may show similar changes in behavior or physiology, and both differ from control females, or PF and PAE offspring may

differ both from each other and from control offspring (Hofmann et al., 1999, Redila et al., 2006, Taylor et al., 1988, Yirmiya et al., 1998). Furthermore, even if behavioral or hormonal responsiveness are similar in PAE and PF offspring, central mechanisms underlying alterations in PAE and PF animals may differ, rather than occurring along a continuum of effects on the same pathway.

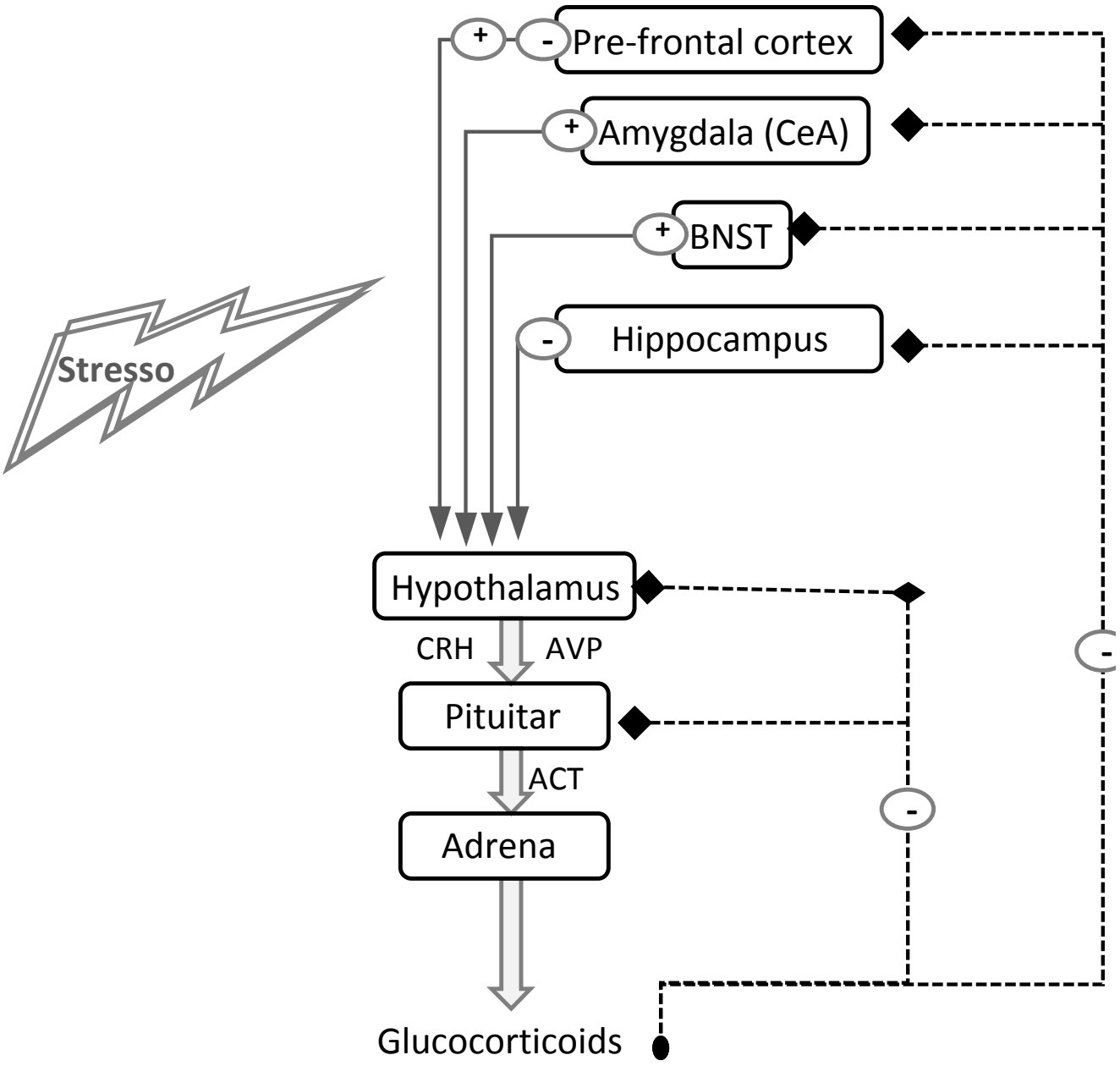
1.5 Stress systems, PAE and vulnerability to stress-related disorders

Both pre-clinical and clinical studies have demonstrated significant alterations in stress regulation following alcohol exposure *in utero* (Haley et al., 2006, Jacobson et al., 1999, Ramsay et al., 1996, Weinberg et al., 2008). Dysregulation of stress systems is known to contribute to a range of mental health problems including substance use disorders (Koob and Kreek, 2007, Koob, 2008, Lovallo, 2006). Two main stress systems include the extra hypothalamic corticotrophin releasing hormone (CRH) system and the locus ceruleus norepinephrine (LC-NE) autonomic system, which result in peripheral effects via the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic system (Schmidt et al., 2008). Both of these systems mediate the effects of external and internal stimuli on stress systems in the brain, and result in physiological responses. The present dissertation focuses on the CRH system and HPA axis. The HPA-axis responds to potential environmental threats to homeostasis (i.e. stressors), and mediates activation of behavioral and physiological responses that facilitate coping (McEwen, 2007). Within the hypothalamus, corticotropin-releasing hormone (CRH) is released in a circadian rhythm from the paraventricular nuclei (PVN), acts on corticotropes within the anterior pituitary,

and stimulates the release of adrenocorticotropic hormone (ACTH) (Figure 1.1). The release of glucocorticoid hormones (cortisol in primates, corticosterone in most rodents) from the adrenal cortex is stimulated by ACTH. Corticosterone (abbreviated as CORT) and cortisol exert significant effects on the central nervous system and body in response to stressors, and provides negative feedback to all levels of the HPA axis and higher brain centers including the hippocampus and prefrontal cortex via actions on glucocorticoid receptors. ACTH produces increases in corticosterone and cortisol levels in both humans and rodents; however the predominate stress hormone following ACTH stimulation is cortisol in humans but corticosterone in rodents.

There are two types of glucocorticoid receptors that are widely distributed throughout the brain's stress systems: 1) The mineralcorticoid receptor (MR) has a high-affinity for CORT (or cortisol) and plays a permissive or tonic role in regulating basal HPA activity, and 2) The glucocorticoid receptor (GR) has a lower-affinity and plays a primary role in suppression of stress-induced HPA activity (De Kloet et al., 1998; Sapolsky et al., 2000). Moreover, MRs and GRs interact, and together play a dynamic role in HPA regulation (Pace and Spencer, 2005; Spencer et al., 1998). Shifts in the balance between MRs and GRs can alter the set point of stress system activity and decrease the ability to maintain homeostasis. In addition, CRH systems interact with effects of glucocorticoids to regulate stress systems in the brain. The extrahypothalamic CRH system includes input from higher brain regions that exert their influence over the hypothalamus (i.e. medial prefrontal cortex, amygdala, bed nucleus stria terminalis, hippocampus) ultimately influencing HPA activity and the secretion of glucocorticoids (Koob and Kreek, 2007, Koob, 2008, Koob and Le Moal, 2008, Lovallo, 2006). It is important to note that measures of central regulation of stress systems are not always

Figure 1.1 Regulation of Hypothalamic-pituitary-adrenal (HPA) axis.



Regulation of Hypothalamic-pituitary-adrenal (HPA) axis. Following the presentation of a stressor, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are two hormones released from the hypothalamus, which in turn stimulate the release of adrenal corticotropin releasing hormone (ACTH) from the anterior pituitary gland. This cascade of events results in the secretion of stress hormones, known as glucocorticoids, from the adrenal cortex. Glucocorticoids can exert their influence on central and peripheral nervous systems. In addition, glucocorticoids provide feedback at all levels of the HPA axis, typically inhibiting further glucocorticoid release and returning the system to homeostasis. However, some brain regions, such as certain subregions of the prefrontal cortex, amygdala and bed nucleus of the stria therminalis, can provide positive feedback in response to high levels of circulating glucocorticoids, and potentiate CRH release from the hypothalamus.

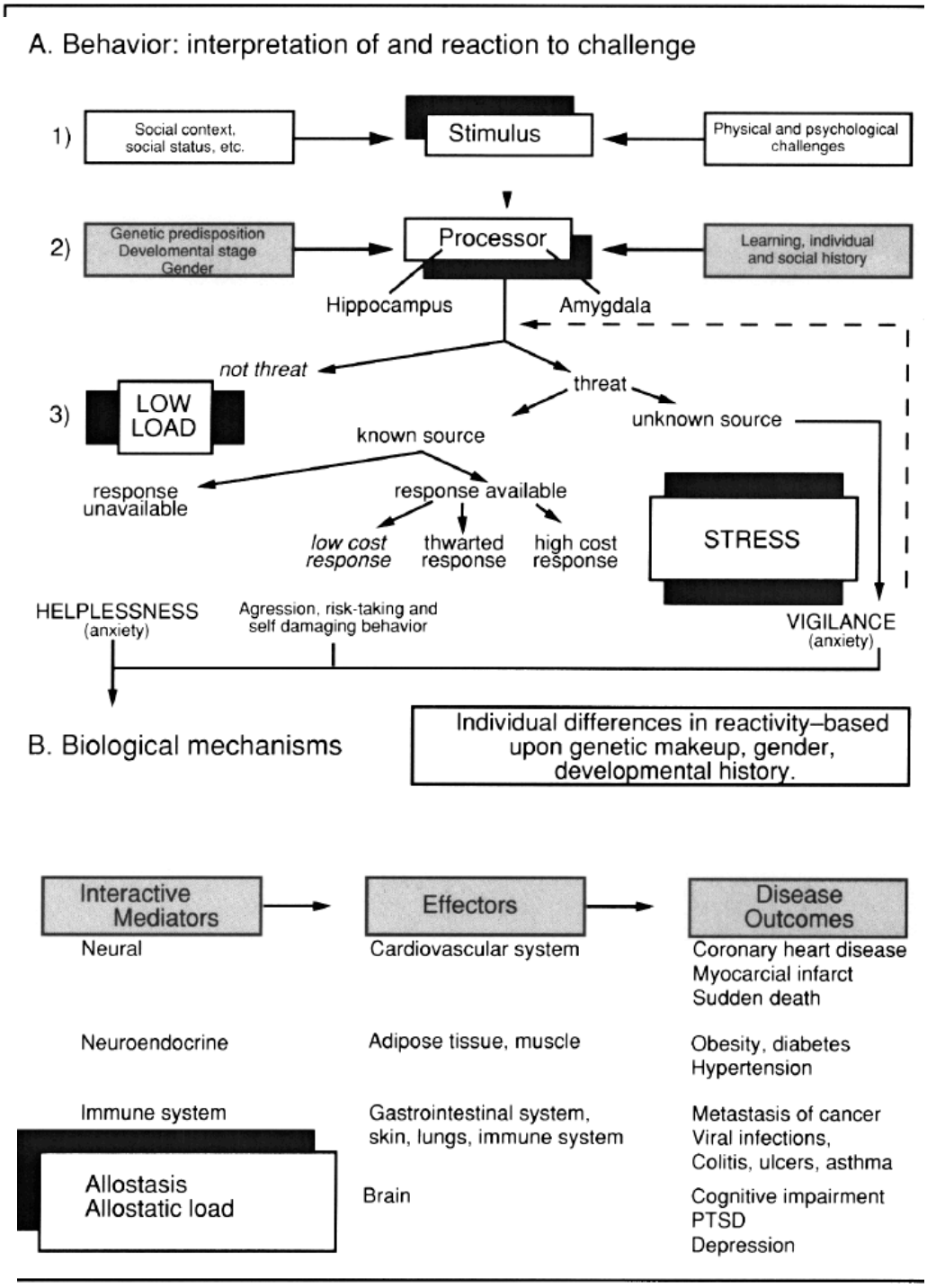
correlated with peripheral hormone levels, and a disconnect between central and peripheral measures may in part underlie vulnerability to mental health problems (de Kloet et al., 2005). For example the interplay among extrahypothalamic regions modulate HPA activity, and widespread alterations in stress systems located within these regions under basal conditions most likely are needed for a vulnerable phenotype to mental health problems, and proceed alterations in peripheral hormone levels (de Kloet et al., 2005). The extrahypothalamic regions are more remote from the hypothalamus, and therefore alterations in basal MR, GR and CRH mRNA expression can contribute to vulnerability to mental health problems in the absence of basal hypothalamic alterations (de Kloet et al., 2005). Further, basal alterations within extrahypothalamic regions may proceed and ultimately contribute to subsequent alterations in: 1) peripheral stress hormones levels in an activated/stress-induced state; 2) cognition; 3) affect; and 4) behavior. In order to elucidate potential neurobiological phenotypes of vulnerability to mental health problems, animal models that combine chronic stress exposure plus a known early life risk factor (i.e. prenatal alcohol exposure) may be particularly useful (de Kloet et al., 2005). Models of acute stress are particularly useful for elucidating alterations in *responding*, while models utilizing chronic stress are more powerful at elucidating alterations in *adaption* (de Kloet et al., 2005).

Dysregulation of stress systems and mental health disorders: Stress responses are adaptive in the short term, utilizing a range of adaptive systems including neural, metabolic and immune functions (McEwen, 2008). However, long-term activation of these short-term adaptive processes often leads to dysregulation of these same systems (McEwen, 2008) including epigenetic modulation (Sinha, 2009). *Allostatic load* develops from a prolonged adaptive response, such as the fight-or-flight response, and may perturb the underlying adaptive systems

leading to the development of pathology (Juster et al., 2010, Lupien et al., 2009, McEwen, 2007), including substance use disorders (Koob, 2008). Continued adjustments in *allostasis* can potentially result in further deviation of the stress responses from baseline functioning, and in turn, alter other systems with which they interact, including dopamine systems (Koob and Le Moal, 2008). Together, basic and clinical studies suggest the long-term neurobiological effects of adversity, during either childhood or adulthood, enhance later susceptibility to substance use disorders (Sinha, 2008, 2009), potentially through an allostatic shift in baseline functioning of stress (Figure 1.2).

The characteristics of the stressor are important in determining whether or not beneficial or detrimental effects on mental health will result. Three key characteristics of a stressor (i.e. predictability, controllability, psychological (versus physical)) have been shown to be particularly influential on potency of stress effects on mental health problems (for review see Miller et al., 2007). Specifically, mental health problems generally follow unpredictable, uncontrollable and psychological stressors (Greenwood and Fleshner, 2008), whereas predictable and controllable stressors can have insignificant or even favorable effects on resiliency. For example, controllability over a stressor has differential effects on stress hormone levels, (Akirav et al., 2001, Prince and Anisman, 1990), neuroplasticity (Kavushansky et al., 2006, Shors et al., 1990), learning (Brown et al., 2001), and even immune function (Laudenslager et al., 1983) compared to those that occur with uncontrollable stressors. In the current thesis, a model of chronic variable stress was implemented (Chapter 2) in order to model unpredictable and uncontrollable chronic psychological stress in our FASD rat model, in parallel with the unpredictable and uncontrollable stressors relevant for the human experience.

Figure 1.2. Process of Accumulation of Allostatic Load



Process of Accumulation of Allostatic Load. A) Behavioral mechanisms that interpret stressful events. 1) A stimulus (or stressor) will initiate different responses depending on the context in which they occur, as well as the specific type of stressor. 2) The process of the stimulus occurs against the individual's biological background. 3) Once processed, the degree of a behavioral response can range from no response, a low cost or high cost response, which depends on the type of response that is available to an individual as well as the ability of the behavioral response to remove an individual from the danger (i.e. low cost response). A high cost response likely involves further confrontation or an inability to escape the stimulus, and includes examples such as substance use or risky physical activity. **B)** Biological mechanisms mediating the behavioral responses include neural, neuroendocrine and immune systems, which ultimately produce an Allostatic load. Adapted from (McEwen, 1998) with permission from Annals of the New York Academy of Sciences (License Number: 2938380566361; Type Of Use: Dissertation/Thesis).

Often CORT is used as a proxy for stress, as stressors typically elevate CORT. Likewise, CORT administration typically mimics the effects of stress. When studying stress hormones as a predictor of vulnerability or resiliency to substance use disorders, the circadian rhythm is also of central importance (Lovallo, 2006). For example, a loss of this circadian pattern of cortisol secretion is related to several mental health problems, including depression and substance use disorders (for review see Duncan, 1996). The timing of stress hormone measurements will significantly impact research findings connecting stress hormone levels with mental health problems. An accurate representation of alterations in stress hormones would require sampling at multiple times to capture true shifts in circadian patterns.

Sexually-dimorphic regulation of stress systems: Significant sex differences exist in the effects of stress, indicating a modulating or mediating role of gonadal hormones (sex steroids). Importantly, the HPA axis does not work in isolation as a large amount of cross-talk between the HPA axis and gonadal hormones occurs. The bi-directional interaction between the hypothalamic-pituitary-gonadal (HPG)- and HPA-axes occurs at multiple levels, where stress typically inhibits HPG function, while sex-steroids reciprocally modulate HPA activity (Young, 1995; Viau, 2002; Weinberg et al., 2008). Due to the reciprocal interaction between the HPG- and HPA-axes, manipulation of one endocrine system will inevitably cause alterations in the other (Viau, 2002). For example, testosterone and CORT (or cortisol) interact at multiple levels of the HPA and HPG axes, with testosterone typically playing an inhibitory role on HPA activity (Viau, 2002). In females, estradiol enhances HPA activity (McCormick et al., 2002), while progesterone functions as a glucocorticoid antagonist via several mechanisms, primarily involving glucocorticoid receptors (reviewed in (Kudielka and Kirschbaum, 2005). Across

species, females typically show greater ACTH responses as well as resistance to negative feedback by glucocorticoids in response to stress compared to males (Young, 1998). Stress-induced alterations within the hippocampus, where a large population of glucocorticoid receptors exist, have been shown to reduce negative feedback of the HPA axis (Goursaud et al., 2006), resulting in a cycle of stress dysregulation. Across species, females consistently exhibit increased basal CORT (or cortisol) levels, which underlie enhanced susceptibility to stress-mediated effects on mental health problems compared to their male counterparts (Becker and Hu, 2008).

Stress systems are dysregulated following in utero alcohol exposure: Many facets of HPA function are altered by PAE. Human and animal studies have shown that PAE programs the fetal HPA axis such that HPA tone is increased throughout life (Weinberg et al., 2008). Basal cortisol levels and cortisol reactivity to stressors are elevated in children exposed to alcohol *in utero* (Haley et al., 2006, Jacobson et al., 1999, Ramsay et al., 1996). Specifically, alcohol-exposed infants display higher basal CORT levels, but a reduced increase in CORT following a stressor compared to non-exposed infants at 2 months, and these alterations were no longer observable at 6 months of age (Ramsay et al., 1996). However, at 5-7 months, alcohol-exposed infants demonstrate increased CORT reactivity, heart rate and negative affect to Tronick's still-face procedure (Haley et al., 2006). By 13 months of age, elevated basal and stress-induced CORT levels are observed (Jacobson et al., 1999). Together, these human studies demonstrate different effects of PAE on basal and stress-induced CORT levels depending on age and stressor type, and show that in general PAE results in elevated basal and stress-induced CORT levels during the first year of life. Moreover, it appears that maturation of HPA responsivity may have a different

developmental trajectory in alcohol-exposed compared to non-exposed infants during the critical first year of life. Similarly, rat models of PAE have shown that PAE offspring exhibit increased HPA activation and/or delayed or deficient recovery to basal levels following stress (Weinberg et al., 2008). Changes in central HPA regulation suggest that PAE results in increased HPA drive and deficits in negative feedback regulation, and likely alters HPA function at multiple levels of the axis (Weinberg et al., 2008). Specifically, enhanced ACTH secretion and CRH mRNA expression are observed in young PAE rats during the first 5 weeks of postnatal life (Lee et al., 1990). However, in adulthood these differences in basal regulation are not readily observed (Kim et al., 1999, Lee et al., 2000), and manipulation of HPA function (via adrenalectomy, dexamethasone administration, or stress exposure) is required to elucidate enhanced HPA drive and diminished negative feedback in PAE animals (Glavas et al., 2001, Osborn et al., 1996, Osborn et al., 2000, Streissguth and O'Malley, 2000). PAE females exhibit enhanced sensitivity of HPA regulation to estradiol levels compared to control females, with PAE females exhibiting higher basal CORT levels during proestrus, but lower basal CORT levels during estrus compared to control females (Lan et al., 2006a, Sliwowska et al., 2008).

Stress systems are dysregulated following in utero alcohol exposure in a sexually-dimorphic manner: Sex-differences in the effects of PAE on HPA-activity are also observed. Following PAE, females tend to show greater HPA responsiveness to acute stressors whereas males show greater responsiveness to chronic or prolonged stressors, although stressor type, intensity and hormonal endpoints measured can all influence the sexually-dimorphic response observed. Importantly, PAE differentially alters HPA activity in male and female offspring (Weinberg et al., 2008). For example, PAE females have an altered balance of receptors

responsible for termination of the stress response (Sliwowska et al., 2008). Similarly, sex differences in HPA, autonomic and behavioral reactivity to stressors have been demonstrated in boys and girls following PAE (Haley et al., 2006), suggesting alterations in the HPA- and HPG-axes interaction following PAE. Further, PAE alters the development of the HPG axis (Esquifino et al., 1986, Lan et al., 2006b, McGivern et al., 1998), as well as HPG-HPA interactions in both male and female offspring (Lan et al., 2009, Lan et al., 2006a, Lan et al., 2006b). For example, testosterone has reduced capacity to regulate HPA function in PAE males (Lan et al., 2009).

1.6 Animal models of life stress

Given the complexity of stress systems and their relationship to mental health problems like substance use disorders, pre-clinical studies are essential for elucidating underlying mechanisms. Many pre-clinical investigations have been conducted with acute stressors, such as restraint or foot-shock. However, these have been criticized for their low construct validity, as it is often the prolonged, unpredictable stress exposure (e.g. work pressure, financial strain) that triggers a depressive episode in humans. As a response to this, the chronic variable stress model (Katz, 1981, Willner, 1997) was developed in an effort elucidate the impact of chronic stress in animals. Often referred to as CUS (chronic unpredictable stress) or CMS (chronic mild stress) as well, this model involves exposing animals to chronic, unpredictable, and psychological stressors, typically over a 4-6 week period, which results in a variety of symptoms that are analogous to those observed in anxiety and depressive disorders (reviewed in (Hill et al., 2008, Redei et al., 2001, Willner, 2005). Specifically, chronic variable stress leads to an increase in

'behavioural despair' (Bielajew et al., 2002, Papp et al., 1994), decreased sucrose consumption (i.e., anhedonia; (Baker and Bielajew, 2007, Baker et al., 2006, Dalla et al., 2008), and an increase in anxiety-like behaviours (D'Aquila et al., 1994, Hellemans et al., 2008). Due to the high co-morbidity between anxiety and depression with substance use disorders (Boschloo et al., 2011, Volkow, 2004), this chronic variable stress paradigm has validity for studying interactions between stress and substance use disorders in rodents. Importantly, evidence indicates that exposure to chronic variable stress results in elevated CORT levels (Herman et al., 2003). Thus, this model appears to reprogram the HPA axis to make the organism more responsive to stressors encountered later in life. In addition, and of relevance to this dissertation, manipulation of the early environment, including repetitive vs. varied prenatal stress, social stress, and maternal deprivation/separation (Caldji et al., 2000, Huot et al., 2001, Ladd et al., 2000, Meaney, 2001, Pryce et al., 2005) also results in neurobehavioral alterations in rodents that resemble those observed with depression, anxiety or substance use disorders in humans. Importantly, prenatal alterations persist into adulthood, suggesting long-lasting effects on underlying neural systems. Importantly, an adapted version of the chronic variable stress paradigm was effective at elucidating anxiety- and depressive-like behaviours in adult male and female PAE rats (Hellemans et al., 2008, Hellemans et al., 2010b). In chapter 2, the effects of chronic variable stress on neural measures within key brain regions known to be important for HPA-dopamine system interactions are explored in PAE males and females.

1.7 Dopaminergic neurocircuitries underlying reinforcement and motivation: sex differences and the effects of PAE.

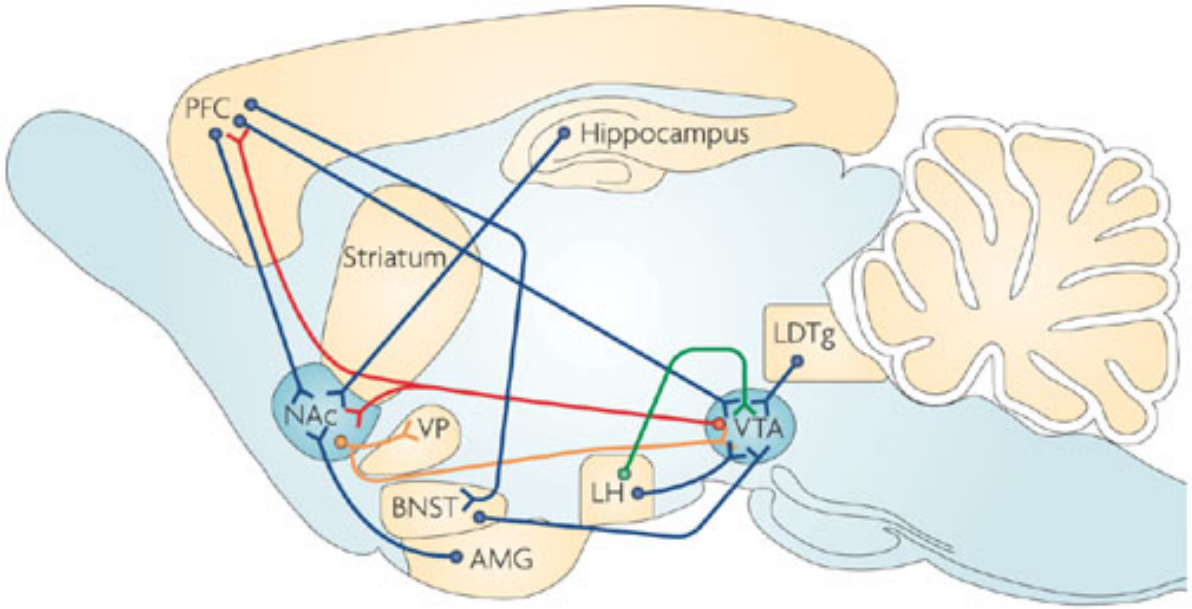
As stress systems become dysregulated, other systems with which they interact can also be significantly altered (Koob and Kreek, 2007). In FASD and PAE models, stress systems are dysregulated and this may contribute to dysregulation in dopaminergic systems. Further, secondary disabilities may include a wide range of mental health problems that add additional challenges which exacerbate the effects of FASD, including alcohol and drug use problems (Alati et al., 2006, Alati et al., 2008, Baer et al., 1998, Baer et al., 2003, Barr et al., 2006, O'Connor and Paley, 2009, Spohr et al., 2007, Streissguth et al., 2004). For example, two different cohorts of individuals exposed to alcohol *in utero* were more likely to present with alcohol use problems themselves; this effect was observed during adolescence and persisted into adulthood (Alati et al., 2006, Alati et al., 2008, Baer et al., 1998, Baer et al., 2003). The increased prevalence of addiction in this vulnerable population is likely influenced by environmental, social, neurobiological, and genetic factors (Li et al., 2001; DeRijk & de Kloet, 2008). Importantly, the studies cited above controlled for important environment and genetic variables such as home environment, parenting styles, and family history of substance use, supporting the hypothesis that *in utero* alcohol exposure results in neurobiological alterations that contribute to enhanced vulnerability to substance use disorders.

Findings in preclinical rodent models of PAE parallel these findings in human FASD populations. For example, it was reported that fetal alcohol-exposed offspring show enhanced voluntary alcohol consumption during both adolescence and adulthood (for review see (Chotro et al., 2007). This evidence further supports the notion that PAE produces neurobiological

vulnerability to substance use disorders throughout life. Neurobiological mechanisms underlying vulnerability to substance use disorders among individuals prenatally exposed to alcohol include: 1) dysregulation of stress systems; 2) alterations in dopamine systems; and 3) alterations in the interaction between stress and dopamine systems. While evidence exists that PAE dysregulates CNS stress systems and alters dopamine systems, to date the effects of PAE on the interactions between stress and dopamine systems have not been fully examined. These current studies were undertaken to elucidate how PAE alters interactions between these systems as a potential pathway to neurobiological vulnerability to substance use disorders in individuals with an FASD. Specifically, the bi-directional interactions between stress and dopamine systems were examined utilizing a rat model of FASD. Chapter 2 describes the effects of chronic variable stress on basal regulation of central stress and dopamine systems in PAE females and males. Chapter 3 describes the differential effects of repeated amphetamine exposure on behavioural sensitization, as well as cross-sensitization with acute stress in PAE females and males, and compared to control counterparts.

In healthy control rodents, stress and dopamine systems interact on multiple levels; therefore dysregulation of one system can lead to dysregulation in the other (Koob and Kreek, 2007). The primary neural circuitry involved in mediating the processes of reinforcement and motivation is the mesocorticolimbic dopaminergic pathway (Figure 1.3). Dopaminergic cell bodies are located in the back of the brain, in the ventral tegmental area, and project to the nucleus accumbens, hippocampus, amygdala, and prefrontal cortex, and underlie neurocircuitries implicated in several functions (e.g. motivation, learning and memory, reinforcement, executive function and emotional regulation). All reinforcers, including both naturally occurring and substance-related, have been found to increase extracellular dopamine levels in the nucleus

Figure 1.3. Schematic of neurocircuitry of the mesolimbic dopamine system



Schematic of neurocircuitry of the mesolimbic dopamine system. In the rat brain, the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA) are highlighted in the following colors: glutamatergic projections, blue; dopaminergic projections, red; GABAergic projections, orange; orexinergic projections, green. Glutamatergic synapses excite, while GABAergic synapses inhibit postsynaptic neurons. Dopamine release from VTA neurons increases in response to administration of all drugs of abuse, as well as in response to novelty. The firing patterns may encode a prediction signaling the reward value of a stimulus relative to its expected value. Abbreviations: AMG, amygdala; BNST, bed nucleus of the stria terminalis; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VP, ventral pallidum. Adapted from (Kauer and Malenka, 2007) with permission to reproduce obtained from Nature Reviews Neuroscience (License Number: 2938380910088; Type Of Use: Dissertation/Thesis).

accumbens and most activate dopamine neurons within the ventral tegmental area (Koob and Le Moal, 2008), making dopamine a central component to reinforcement. Substances with abuse potential are typically more potent at evoking dopamine release than naturally occurring stimuli, making them generally more effective at inducing neurobiological alterations than naturally occurring reinforcers.

Conditioned cues associated with natural reinforcers, such as predictors of desirable food, evoke dopamine release from the nucleus accumbens core, whereas drug-conditioned stimuli preferentially evoke release from the nucleus accumbens shell (Rouge-Pont et al., 1995); therefore optimally positioned for further dysregulation of additional dopamine systems (Di Chiara and Bassareo, 2007). The hippocampus initially helps make drug-cue associations, and then plays a role in 'gating' or monitoring drug stimulated inputs from the nucleus accumbens depending on previously acquired drug-cue information (Grace et al., 2007). The prefrontal cortex is involved in monitoring 'motivational salience', by influencing nucleus accumbens outputs via excitatory inputs from the prefrontal cortex to the nucleus accumbens (Kalivas et al., 2005). The hippocampus modulates input from the prefrontal cortex at the level of the nucleus accumbens (Grace et al., 2007). An additional dopaminergic system that is readily recruited by drug-conditioned stimuli is the nigrostriatal dopaminergic pathway (substantia nigra to the striatum), which is primarily involved with the motor effects of substances with abuse potential. The nigrostriatal dopaminergic pathway receives efferent projections from the nucleus accumbens and is thought to play a more prominent role in substance related reinforcement and conditioning following repeated substance use, rather than acute use.

Dysregulation of dopamine systems and vulnerability to substance use disorders: In addition to dysregulation of stress systems, dysregulation of dopamine systems is of central importance for resilience against, or vulnerability to, substance use disorders (Di Chiara and Bassareo, 2007, Everitt et al., 2008, Le Moal, 2009, Sinha, 2008, Sweitzer et al., 2012, Volkow et al., 2011). A large degree of neuroadaptation occurs in response to substance use, and these neuroadaptations set the stage for increased propensity to develop subsequent disorders associated with substance use (Koob and Le Moal, 2008, Russo et al., 2009) as well as increased vulnerability to relapse after prolonged periods of abstinence (Robinson and Berridge, 1993, 2008). For example, altered dendritic branching and density within the prefrontal cortex and nucleus accumbens can be seen following repeated injections of various drugs with abuse potential in male rats (Robinson et al., 2002). In humans, males with a former substance use problem continue to demonstrate alterations in brain activity even after prolonged abstinence. Using positron emission tomography (PET) imaging studies, men exhibited significantly decreased dopaminergic receptors (D_2) and stimulated dopamine release in the striatum and nucleus accumbens compared to men without a history of substance use disorders (Volkow et al., 2009). This decrease in dopamine receptors and release was correlated with decreased levels of regional activation in key brain areas involved in saliency attribution, control of inhibition, and executive functioning even after 4-6 months of abstinence compared to healthy controls (Volkow et al., 2009). Interestingly, neural changes result from chronic use of a broad range of substances, including alcohol, caffeine, nicotine, heroin and cocaine (Koob and Kreek, 2007, Russo et al., 2009, Volkow et al., 2009). This indicates that there are common substance-induced neurobiological alterations, which may produce a universal neurobiological pathway to increased vulnerability to subsequent substance use disorders.

A current theory of substance use disorders is that problematic substance use parallels an imbalance between dopamine circuitries underlying executive functioning and decision making with those underlying substance-related reinforcement and conditioning (Everitt et al., 2008, Volkow et al., 2011). For example, dopamine responses in individuals with substance use disorders are blunted in response to substance administration, but enhanced in response to substance-conditioned stimuli in the absence of the substance itself (Volkow et al., 2011). Accumulating evidence suggests that problematic substance use is correlated with reduced executive function and emotional regulation, supporting the theory of an acquired drug-induced imbalance of dopamine systems (Di Chiara and Bassareo, 2007). Indeed, individual differences in certain cognitive functions, such as impulsivity, may be a result of inherent differences in dopamine function, possibly related to genetic polymorphisms (Le Moal, 2009, Sweitzer et al., 2012). For example, reduced binding of type 1 and 2 dopamine receptors (D_1 and D_2), as well as functional polymorphisms of their genes, have been associated with increased susceptibility to substance use disorders (Hooks et al., 1994, Sweitzer et al., 2012, Volkow et al., 1999, Volkow et al., 2011). Reduced binding of dopamine receptors (D_1 and D_2) within the striatum and nucleus accumbens is also correlated with attenuated baseline activation of the prefrontal cortex (orbitofrontal cortex) in humans, but augmented activation in response to drug-conditioned stimuli compared to controls (Heinz et al., 2004, Volkow and Fowler, 2000, Volkow et al., 2011). Unfortunately, neuroimaging studies assessing alterations in dopaminergic activity have not been conducted with individuals with FASD. Therefore, parallels cannot be directly drawn between FASD studies and other human neuroimaging studies on substance use disorders and represents a gap in the literature. Thus, dopamine receptor expression is measured in studies presented in chapters 2 and 3, and are integrated with past literature in the discussion.

Sex differences in substance use disorders: Sex differences are observed in substance use disorders, as well as with additional mental health problems (Becker and Hu, 2008). Sex differences in stress and dopamine systems, suggests that the HPG axis also plays a role in vulnerability to substance use disorders. Further, past research in both the substance use disorders and stress fields provides evidence for sex differences in behavioral responses and neurobiology associated with each (Becker and Hu, 2008, Haseltine, 2000, Lynch et al., 2002, Roth et al., 2004, Wetherington, 2007). For example, motivation to use substances may differ between sexes: typically, men use substances for the 'rewarding' properties while women aim to relieve stress with their substance use (Becker and Hu, 2008, Haseltine, 2000). Both clinical and basic research suggests that females tend to escalate their consumption of substances more quickly and at lower doses, and are more prone to stress-induced relapse than males (Haseltine, 2000, Hu and Becker, 2003), especially when levels of circulating estradiol are increased (Becker and Hu, 2008). Although it is probable that psychosocial factors have an impact, converging evidence from animal studies suggest that fluctuating ovarian hormones play an important role in these differences. In rats, cocaine self-administration varies across the estrous cycle (Roberts et al., 1989), and high doses of estradiol facilitate cocaine self-administration (Lynch et al., 2001). Moreover, the enhanced acquisition of cocaine self-administration in female rats is abolished in ovariectomized animals, but can be reversed with administration of estradiol (Hu et al., 2004). Both clinical and basic research suggests that females tend to escalate their consumption of drugs more quickly and at lower doses, and are more prone to relapse than males (Becker and Hu, 2008, Haseltine, 2000, Hu and Becker, 2003). These data suggest that estrogens facilitate dopaminergic function.

However, the nature of sex differences in vulnerability to substance use disorders is not fully understood. Despite the evidence suggesting that females are more vulnerable to substance use disorders on a neurobiological level, substance abuse and dependence disorders are 2 to 3 times more likely in men than women in adulthood (Van Etten and Anthony, 2001). These contradictory findings may reflect the differences in social surroundings between men compared to women, which ultimately determine one's exposure to substance use (Becker and Hu, 2008). However, another possibility is that fluctuating hormone levels during the menstrual cycle may produce significant changes in drug-seeking/wanting cognitions; therefore changing the perceptual experience surrounding substance use, and may be a potential protective factor at times. However, steadily increasing rates of illicit and non-illicit drug use in young girls compared to adolescent boys suggests that this statistic could be changing (Becker and Hu, 2008). Social factors can interact with and/or override biological factors; therefore, making animal models useful for investigating neurobiological underpinnings of vulnerability to substance use disorders while controlling the social environment. Thus, sex differences in underlying plasticity of dopaminergic systems, as well as alterations in gonadal hormones will be examined in Chapters 2 & 3.

Alterations in dopamine systems following PAE: In addition to substance use disorders, dopaminergic function also influences a range of mental health and cognitive problems occurring at an increased prevalence among individuals with FASD, such as difficulty with inhibitory control, anxiety, depression, attention deficit disorder, schizophrenia and conduct disorders (Mihalick et al., 2001, O'Connor and Paley, 2009, Pei et al., 2011, Rasmussen, 2005). Importantly, there is evidence that PAE produces significant alterations in dopamine systems

(Blanchard et al., 1993, Carneiro et al., 2005, Spear, 1996, Xu and Shen, 2001). Development of dopaminergic systems was delayed by prenatal exposure to moderate levels of alcohol via a liquid diet throughout gestation; however, this effect was only observed in young male pups, whereas all females overall displayed delayed maturation compared to males regardless of PAE (Hannigan, 1990, Hannigan et al., 1990). These sex-related differences may be mediated via an interaction between hormones and dopamine function. Indeed, using the dopamine (D_2) antagonist sulpiride, Hannigan and colleagues (Hannigan et al., 1997) observed a profound dose dependent increase in prolactin levels in females exposed to varying levels of PAE from gestational day 8 to 20 (with the highest level of alcohol having the greatest effect).

Although the majority of studies in PAE animals do not find alterations in number of dopamine receptors, one study found a moderate increase in D_1 receptors (5–10% increase) in the striatum on postnatal day 19 in PAE pups (Gillespie et al., 1997). One mechanism by which PAE may change dopamine systems is by altering dopamine neurons. Reduced cell body size, stunted growth of dendrites, and diminished electrical activity of dopamine neurons have been reported in PAE animals (Choong and Shen, 2004a, Choong and Shen, 2004b, Kaneko et al., 1993, Shen and Choong, 2006, Shen et al., 1999, Shetty et al., 1993, Wang et al., 2006, Xu and Shen, 2001). For example, PAE altered sensitivity of pre-and postsynaptic dopamine receptors (Hannigan, 1996, Shen et al., 1999), but only under certain conditions in PAE females (Hannigan, 1990). Additional aspects of dopamine systems were found to be decreased by PAE including dopamine synthesis, binding sites of receptors, dopamine uptake sites, and metabolites of dopamine (Cooper and Rudeen, 1988, Druse et al., 1990, Randall and Hannigan, 1999, Rathbun and Druse, 1985, Szot et al., 1999), with some of these effects fading in adulthood

(Middaugh et al., 1994). Together, these studies support the idea of reduced overall baseline activity of dopamine systems.

Reduced activity is observed in young and older male rats following PAE, and is not due to a reduction in dopamine neuron numbers, firing rates or patterns, indicating the presence of a depolarization block, rather than enhanced inhibition in PAE animals (Shen et al., 2007, Shen et al., 1999, Xu and Shen, 2001). The enhanced inhibition of tonic dopaminergic activity could result from either excessive excitation or from alternative inputs to dopamine neurons within the ventral tegmental area (Shen and Choong, 2006). Interestingly, the activity of the dopamine neuron population within the ventral tegmental area resembles that previously observed in chronic antipsychotic treated animals (Grace et al., 1997, Shen et al., 2007), and repeated exposure to a low dose of a central nervous system stimulant (i.e. amphetamine or methylphenidate) was able to reverse the depolarization block, restoring basal dopaminergic activity in PAE animals (Choong and Shen, 2004a, Shen and Choong, 2006, Xu and Shen, 2001).

PAE rodent models have clearly demonstrated altered function of key neurotransmitters important in reinforcement and motivation following PAE using a variety of methodologies. When pharmacologically challenged, adult PAE animals appear to have sustained alterations in dopaminergic function. Interestingly, PAE typically attenuates behavioural effects of dopamine antagonists, but enhances effects of dopamine agonists (Barbier et al., 2009, Becker et al., 1993, Blanchard et al., 1993, Hannigan, 1996, Hannigan et al., 1990, Nowak et al., 2006). PAE reduced sensitivity to alcohol sedation using a 'loss of righting reflex' method, which was associated with enhanced preference for and consumption of alcohol (Barbier et al., 2009). Regarding stimulants, PAE *enhanced* behavioral sensitization following acute and repeated stimulant exposure (Barbier

et al., 2009), suggesting that some degree of endogenous sensitization of dopamine systems is occurring without alterations in quantity of dopamine neurons, content or receptors. Importantly, altered motor responses to drugs are thought to be indicative of alterations in the neurocircuitry underlying reinforcement and motivation. Specifically, heightened behavioural (locomotor) responses to stimulants are positively correlated with increased drug consumption in control animals, as well as reflective of underlying alterations in neurobiology involving dopamine and glutamate. Interestingly, the effect of PAE on responsiveness to stimulant-enhanced locomotion varies depending on the age at testing and sex of the rat (Blanchard et al., 1987, Hannigan and Pilati, 1991a), but in general PAE results in enhanced responsivity to stimulants.

Although the majority of studies found enhanced sensitivity to stimulants, neurochemical studies suggest attenuation of stimulant-induced dopamine release in PAE animals. One study found that PAE did not alter amphetamine-evoked changes in dopamine levels in adulthood, unless paired with prenatal exposure to another central nervous system teratogen (e.g. cadmium) (Nowak et al., 2006). PAE augmented alcohol-induced increases of dopamine in the nucleus accumbens and striatum in males, but attenuated dopamine release in the striatum of PAE females (Blanchard et al., 1993). Additionally, PAE did not alter basal concentrations of dopamine, and attenuated cocaine-evoked increases in dopamine levels in males and females on postnatal day 10 (Chen et al., 1997). Importantly, alterations in behavioral responsivity to dopaminergic drugs following PAE occurs in a dose-dependent manner, particularly following stimulation of dopaminergic pathways (Hannigan and Berman, 2000, Middaugh et al., 1994), with the highest doses of PAE resulting in the greatest behavioral alterations.

1.8 Modeling substance use disorders in animals

As mentioned previously, pre-clinical research is needed to elucidate how PAE alters underlying neural systems implicated in the etiology of substance use disorders. In humans, a diagnosis pertaining to substance use disorders relies on a blend of observations and self-reports. Hallmarks of substance use disorders (i.e. addiction) include: 1) an increasing intake of substance(s); 2) uncontrollable desire to use and/or unsuccessful efforts to reduce/control use; 3) high expenditure of time in drug-seeking/taking behavior; 4) failing to meet important obligations, reducing previously enjoyed activities; 5) continued use despite adverse consequences; 6) a high tolerance; and 7) withdrawal symptoms with increasingly shorter periods of abstinence. As many of these human characteristics are difficult to model in animals, research on substance use disorders has focused on developing models that imitate individual components of addictive-like behavior.

PAE rodent models of substance use disorders and dopamine: For the most part, the link between PAE and addictive-like behaviors in rodent models has been limited to the investigation of enhanced preference for and consumption of alcohol later in life (Chotro et al., 2007), with little investigation of preference for additional substances with abuse potential. Nonetheless, these studies have been important in showing an enhanced susceptibility for addictive-like behaviour following PAE. For example, alterations in alcohol consumption patterns have been studied as a marker of addictive-like behaviour, and a number of studies have manipulated timing, pattern, and dose of PAE, as well as genetics (Chotro et al., 2007) to further elucidate potential mechanisms. Studies by Milana and colleagues (Chotro et al., 1991, Dominguez et al.,

1998, Molina et al., 1995, Molina et al., 1987), as well as Arias and Chotro (Arias and Chotro, 2005b), have shown that repeated alcohol exposure late in gestation (gestational days 17-20) increases later consumption and alcohol-related learning in pups. Although acute exposure is not as widely investigated, a single day of alcohol exposure (gestational day 8) also produced increased consumption (Molina et al., 1987). Interestingly, although exposure during gestation in rodents is sufficient to augment alcohol consumption in pups, additional postnatal exposure (equivalent to the third trimester of pregnancy in the human) appears to enhance this effect (Holloway and Tapp, 1978). However, a few animal studies did not find increased alcohol consumption following PAE (Abel and York, 1979, Grace et al., 1986b, McGivern et al., 1984, Reyes et al., 1985) and these discrepancies are likely due to timing and dose of alcohol exposure *in utero*, the age and sex of the offspring at testing, and the alcohol consumption paradigm utilized (Chotro et al., 2007). Although increased consumption may or may not indicate an enhanced preference for the substance, a number of studies indicate this may indeed be the case. Using the conditioned place-preference paradigm to assess preference for alcohol, animals exposed to alcohol during either prenatal or early postnatal periods show enhanced preference for alcohol (Barbier et al., 2009, Pautassi et al., 2012). Additionally, enhanced preference for alcohol odor was found after an acute administration of alcohol in the amniotic fluid (gestational day 21) and after repeated maternal intragastric intubation (2 grams/kilogram) from gestational days 17 to 20 (Abate et al., 2002, Chotro et al., 1991). It is important to note the studies discussed above used intragastric administration of alcohol to pregnant dams, increasing the difficulty in separating the effects of alcohol alone from the potential interaction of alcohol plus maternal stress on the developing fetus. However, a number of other routes of administration have been shown to enhance alcohol consumption, such as alcohol-containing liquid diet or

replacement of water with an alcohol/water mixture (Bond and DiGiusto, 1978, Buckalew, 1979), which are thought to produce less maternal stress.

In addition to timing and mode of administration, a range of doses and types of alcohol (e.g. ethanol, wine, beer) have been used to demonstrate increased consumption in the offspring of alcohol exposed dams (Chotro et al., 2007), including doses as low as 1-2 g/kg (Arias and Chotro, 2005a, c, Chotro and Arias, 2003, Dominguez et al., 1998, Molina et al., 1987). Moreover, increased alcohol consumption has been demonstrated at various ages ranging from postnatal day 14 to 170, and utilizing various types of alcohol (e.g. wine, beer, ethanol with or without sweetener) (Chotro et al., 2007, Dominguez et al., 1998, Lancaster and Spiegel, 1989, Molina et al., 1995, Phillips and Stainbrook, 1976). Although owing to variations in methodology, a few studies have reported no prenatal alcohol-induced increase in later alcohol consumption (Abel and York, 1979, McGivern et al., 1987), the majority of findings suggest a relatively permanent effects of PAE on enhanced consumption (Dominguez et al., 1998, Grace et al., 1986a). Characteristics associated with substance use disorders, such as novelty-seeking and impulsive behaviours, further elucidate mechanisms in rodents. Decreased behavioural inhibition is a fundamental characteristic of substance use disorders, and alterations have been demonstrated in FASD rat models using a two-way shuttle avoidance learning task (Bond and DiGiusto, 1978, Rockwood and Riley, 1985).

PAE rodent models of SUDs: dopamine and other key neurotransmitters: Although dopamine systems are strongly implicated in etiology of substance use disorders, and are significantly altered by PAE in males and females, opioid and glutamatergic systems are also of importance in understanding the propensity towards substance use disorders in models of FASD.

For instance, increases in alcohol preference in prenatally alcohol-exposed rats was blocked by the co-administration of an opioid receptor antagonist, naltrexone (Arias and Chotro, 2005b). Administration of nalaxone (similar to naltrexone) resulted in decreased opioid binding in the striatum of PAE pups compared to control pups (Shah and West, 1983). Additionally, glutamatergic function appears to be altered by PAE, as evidenced by altered expression and binding of receptors (Honse et al., 2003a, Honse et al., 2003b, Nixon et al., 2002). Although these neurotransmitter systems are altered by PAE, and are known to play a role in vulnerability to substance use disorders, they are outside of the scope of the present dissertation and are not examined further.

Importance of genetics and maternal stress: As mentioned, both genetics and stress likely contribute to the increased propensity to develop substance use disorders among individuals with an FASD. Although these areas have not been studied extensively, novel research is perhaps beginning to elucidate their contribution. For example, genetic contributions have been investigated using alcohol preferring (AA: Alko Alcohol) and alcohol-avoiding (ANA: Alko Non-alcohol) rat lines, where PAE increased later consumption in ANA offspring only (Hilakivi et al., 1987).

Limitations of PAE rodent models of substance use disorders: At present, the accumulation of substance use disorders and FASD rodent studies supports the hypothesis that PAE enhances risk for the development of substance use disorders. However, this hypothesis remains to be fully elucidated within FASD rodent research. Particularly, a significant gap exists between the large number of paradigms available for rodent models of substance use disorders

and those that have been implemented with FASD rodent models. The human experience of substance use disorders is centered on 'problematic' use, and insightful drug use paradigms can elucidate aspects of problematic substance use in rodents, yet have not been employed with FASD rodent models. This is likely due to the feasibility of executing study with substances other than alcohol, such as the self-administration paradigm, with a large number of offspring.

PAE non-human primate models of substance use disorders: The use of non-human primates to study substance use disorders following PAE has been restricted due to problems related to maternal alcohol administration, the high expense and longevity of experiments, as well as the small number of offspring per mother (typically a single offspring). Nonetheless, the few primate studies that have been conducted have been highly valuable in further linking PAE to risk for substance use disorders in an FASD animal model. A prominent focus of non-human primate (rhesus monkey (*Macaca mulatta*)) work has been to separate effects of prenatal stress from those of early alcohol exposure, to address the question of whether environmental factors, such as stress, augment the effects of PAE (Schneider et al., 2002). For example, 'stereotypies' refer to a class of behaviours indicative of underlying mental pathology related to vulnerability to substance use disorders. Monkeys prenatally exposed to alcohol plus stress show higher levels of stereotypies compared to those exposed to stress only or alcohol only, and compared to control monkeys (Schneider et al., 2001, Schneider et al., 2002). Thus, stress appears to enhance the behavioural effects of fetal alcohol exposure.

Importantly, the non-human primate FASD model has also provided support for prenatal alcohol-related alterations in dopamine systems. For instance, PAE increased dopamine synthesis and receptor binding in primates exposed to alcohol and/or stress prenatally (Schneider et al.,

2005). Importantly, these underlying alterations in dopamine systems has been correlated with relevant behaviours related to substance use disorders, like alterations in behavioural inhibition following PAE (Roberts et al., 2004). As with the rodent model, timing of exposure remains an important factor. For example, in the Pigtail Macaque (*Macaca Nemestrina*), baseline dopamine concentrations within the striatum were increased by weekly exposure to alcohol throughout gestation and if exposure to alcohol was delayed to the fifth week of gestation, there was a negative correlation between maternal peak plasma alcohol concentrations and dopamine concentrations, with higher maternal peak plasma alcohol concentrations relating to lower striatal dopamine concentrations (Clarren et al., 1990). These findings suggest that alterations in dopaminergic function caused by PAE are different depending on the onset of alcohol exposure during gestation.

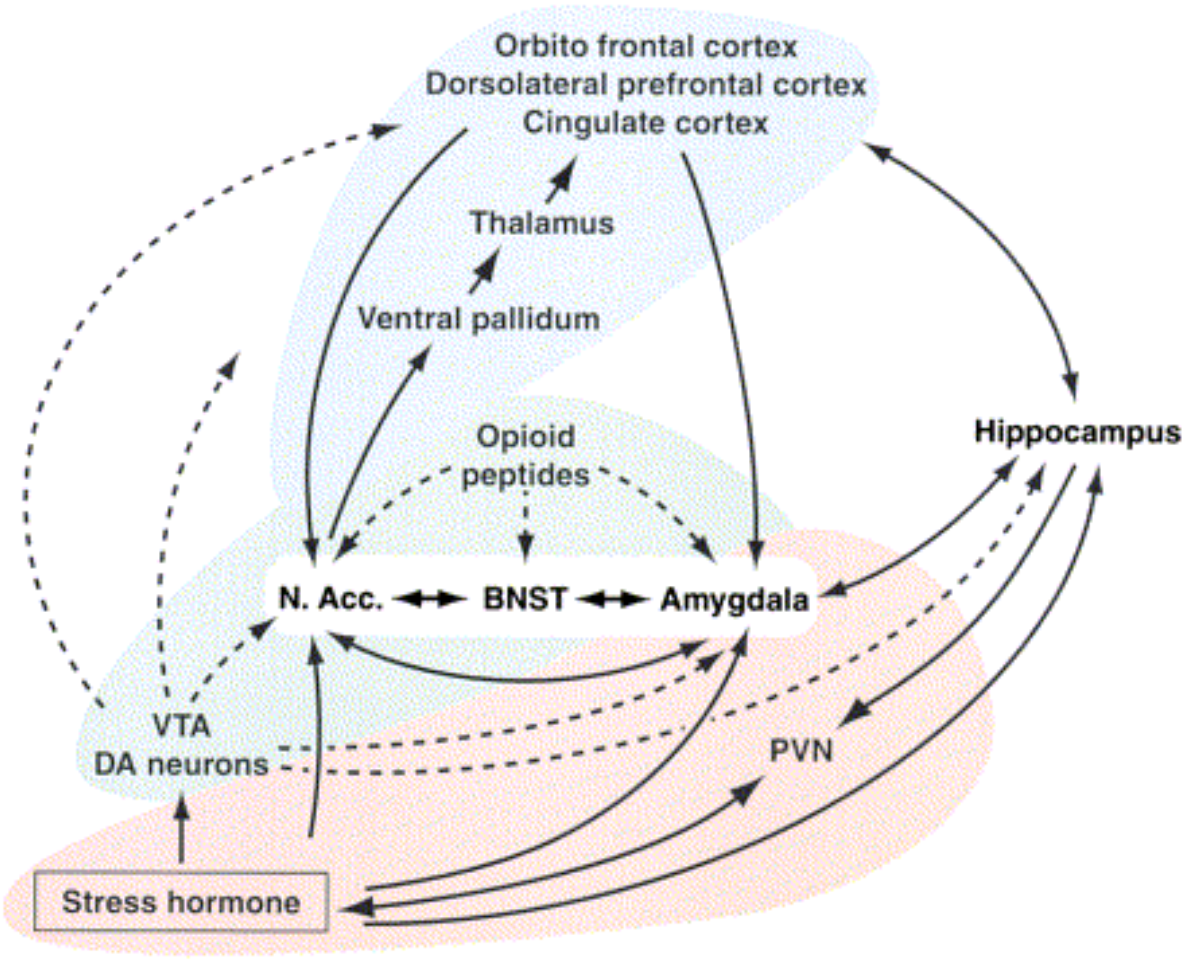
Overall, findings from PAE models indicate enhanced vulnerability to substance use disorders: Findings from rodent and non-human primate models of FASD consistently support the hypothesis that PAE enhances risk for the development of substance use disorders. Despite numerous elegant studies with FASD animal models and substance use disorders, there is a large gap between techniques available to study addictive-like behaviour in animals with what has been investigated in FASD studies. Innovative methodologies are continually being created and provide novel ways to view 'addictive-like' behaviour in animals. Therefore, there is the potential to close the gap between what is available and what is being implemented in FASD animal models. Additionally, research on substance use disorders is mainly executed in the male rat, and the vast amount of knowledge has not been examined in the female. Fortunately, a prominent strength of most FASD animal studies has been the inclusion of both sexes; therefore,

findings from the FASD and substance use disorder fields may lend valuable insight to the larger field of substance use disorders specifically pertaining to the female. Elucidation of the role of HPA and gonadal hormones in vulnerability to substance use disorders can provide valuable information for interventions and treatments not only for individuals with FASD, but to the general public overall. Additional considerations exist when incorporating female rodents into studies, such as assessing stage of estrous and differential developmental time periods.

1.9 Interactions between stress and dopamine systems

Importantly, there is significant overlap between the extrahypothalamic CRH systems and dopaminergic pathways (Cabib and Puglisi-Allegra, 2012). This is important for the current dissertation because PAE alters stress and dopamine systems, and an increased prevalence of substance use disorders occurs among individuals with a FASD, and in typical control subjects the interaction between stress and dopamine plays a role in vulnerability to substance use disorders. This results in a bi-directional interaction between stress and dopamine, allowing for between systems adaptations, where changes in one system result in changes in another system (Koob and Kreek, 2007) (Figure 1.4). For example, the position of the bed nucleus stria terminalis allows for integration of information from stress and dopamine systems, while the hippocampus, medial prefrontal cortex, and amygdala are directly involved in both systems. Both stress and acute drug use can increase extracellular dopamine in the nucleus accumbens shell (Rouge-Pont et al., 1995) and excitability of dopamine neurons within the ventral tegmental area (Saal et al., 2003). Efferent and afferent projections of the extended amygdala (i.e. central

Figure 1.4. Neurobiology underlying interaction between substance use and stress



Neurobiology underlying interaction between substance use and stress. Above is a simplified illustration of the neurobiological interaction between the mesocorticolimbic dopaminergic neurocircuitry and the extrahypothalamic stress systems. In brief, circulating stress hormone (glucocorticoids) exerts direct effects on brain regions involved in reward (ventral tegmental area (VTA), nucleus accumbens (NAc.), amygdala (CeA), Hippocampus (HIPC)) and modulates dopamine activity. Reciprocally, dopamine transmission within these key brain regions modulates HPA function. Thus, stress and substance use have a bi-directional influence on underlying neurobiological systems. Adapted from (Koob and Le Moal, 2001) with permission obtained from Neuropsychopharmacology.

nuclei of the amygdala plus the bed nucleus stria terminalis) include the hypothalamus, allowing significant influence over HPA activity (Lovallo, 2006), with substance use increasing stress responsivity in rodents. The medial prefrontal cortex attenuates nucleus accumbens innervations, and lesions within the medial prefrontal cortex result in heightened behavioral responses to stress and heightened stress-induced dopamine release within the nucleus accumbens (Brake et al., 2000). Following extinction of drug-taking behavior, stress was found to be more effective at reinstating drug-taking behavior than priming with drug itself (Shaham et al., 1996, Shaham and Stewart, 1996). Prolonged exposure to glucocorticoids can have opposing effects (inhibition of dopamine synthesis) compared to an acute stressor (facilitation of dopamine synthesis) (Pacak et al., 2002). Basal corticosterone levels in non-dependent rats positively correlate with the degree to which an animal will self-administer drug. CRH is released with escalation of drug intake during self-administration in rodents (Sarnyai et al., 2001). Similar to substances with abuse potential, dysregulation of the HPA axis has been observed with chronic alcohol consumption in both rats and humans. HPA activity increases with acute alcohol consumption, but decreases with repeated exposure (Lee et al., 2001). Animal models have illustrated differences in HPA responsivity to drug use in non-dependent compared to dependent animals. For example, *activation* of the HPA-axis is observed in non-dependent animals, however with prolonged use, this relationship becomes significantly altered, where withdrawal *activates*, and drug use *attenuates* HPA-activity (Koob and Le Moal, 2008). Importantly, this bi-directional relationship between stress and substance use has been demonstrated with a variety of stressors as well as a variety of substances (Ahmed and Koob, 1997, Buczek et al., 1999, Le et al., 2000, Shaham et al., 2000), indicating common underlying stress pathways are linked with substance use.

Evidence of stress and dopamine interactions in humans: Similarly in humans, stress sensitizes (response to the substance is increasingly amplified) healthy individuals to the rewarding effects of substance use, and can induce relapse after abstinence (Sarnyai et al., 2001), further suggesting that HPA alterations provide a pathway for increased vulnerability to substance use disorders and relapse. Individuals with alcoholism going through acute withdrawal have elevated CRH levels in their cerebrospinal fluid (Adinoff et al., 1996b). Further, increased alcohol consumption, craving and relapse to drinking have been reported in abstinent individuals with alcoholism exhibiting high levels of stress and anxiety (Kushner et al., 1990). Periods of heavy substance use can result in chronic activation of the HPA axis (Lovallo, 2006), which may lead to deficits in normal HPA-feedback. Individuals with alcoholism going through acute withdrawal have elevated CRH levels in their cerebral spinal fluid (Adinoff et al., 1996a). Chronic alcohol consumption also results in a loss of diurnal rhythm of cortisol secretion, manifest as an overall decrease in cortisol compared to healthy controls, which may be regained 1-4 weeks into abstinence (Adinoff et al., 2005). However, the magnitude of the decrease in cortisol secretion following cessation of use can be predictive of propensity to relapse, suggesting individual differences in vulnerability to relapse directly related to HPA dysregulation (Lovallo, 2006). Interestingly, human subjects with a positive family history of substance use disorders exhibit alterations in HPA-activity in the absence of a history of substance use (Wand et al., 1998), suggesting that alterations in the stress response may underlie substance use disorders in this particular population. Attenuated HPA responses found in abstinent individuals with alcoholism may be reflective of a preexisting difference in baseline HPA-activity (Lovallo, 2006). The literature undoubtedly argues that stress and substance use disorders are intimately related, where alterations in HPA-activity may lead to vulnerability to

substance use disorders and relapse. However, it is not yet clear as to what predisposes an individual to alterations in sensitivity to dysregulation of stress and/or dopamine systems.

Importance of subregions of the prefrontal cortex, nucleus accumbens, bed nucleus of the stria terminalis and hippocampus in HPA-dopamine interactions: Within the overlapping neurocircuitry of stress and dopamine systems, it is important to note that subregions play unique, and sometimes opposing, roles within each encompassing brain region. For example, the prelimbic and infralimbic subregions together make up the medial prefrontal cortex, and each subregion uniquely regulates activation of stress systems. CRH expression in the prelimbic subregion typically inhibits, while the infralimbic potentiates, activation of brain stress systems (Sullivan and Gratton, 2002). In the bed nucleus stria terminalis, CRH mRNA levels in the anterior bed nucleus stria terminalis increase, while levels in the posterior decrease, CRH mRNA expression the paraventricular nucleus of the hypothalamus (Herman et al., 1994). Subregions of the hippocampus (i.e. dentate gyrus, cornu ammonis areas 1-3) are also known to be affected differentially by chronic stress (Kavushansky et al., 2006), and therefore likely play unique roles over stress circuitry, although roles of specific subregion are not fully understood.

1.10 Specific aims and hypotheses

Evidence from past studies supports the hypothesis that dopamine-stress interactions are altered by PAE (Blanchard et al., 1993, Nelson et al., 1983, Taylor et al., 1986). Alcohol-exposed neonates (i.e. postnatal day 5-7) show blunted HPA responsivity to alcohol and

morphine challenges compared to control neonates (Taylor et al., 1986), but augmented HPA responsivity to alcohol and morphine in adulthood (Nelson et al., 1983, Taylor et al., 1982, Taylor et al., 1981). Additionally, PAE enhanced stress-induced alcohol consumption (Nelson et al., 1983). Given the effects of PAE on both stress and dopamine systems, it is important to elucidate the effects of PAE on interactions between these systems in order to better understand neurobiological vulnerability to SUDs in this population. In Chapter 2, the PAE effects on stress signaling and dopamine receptor expression were examined in key brain regions where stress and dopamine systems intersect. CRH mRNA, as well as glucocorticoid, cannabinoid and dopamine receptor expression were measured under both no-stress and chronic stress conditions. Sex differences in outcome were also investigated as: 1) there are sex differences in HPA function across species, with females typically showing greater HPA responses and resistance to negative feedback compared to males (Young, 1998); 2) PAE differentially alters HPA activity in males and females (Haley et al., 2006, Weinberg et al., 2008); and 3) sex differences are observed in SUPs (Becker and Hu, 2008, Haseltine, 2000).

Given the links between stress and DA systems, and the effects of PAE on these systems, **I hypothesized that PAE will alter the effects of chronic stress on the brain, and that sex differences will be present in the effects of both PAE and stress.** In Chapter 3, the effects of PAE and repeated amphetamine exposure on behavioral sensitization, as well as cross-sensitization with HPA function are examined in both female and male rats. **I hypothesized that PAE will 1) augment behavioral sensitization to amphetamine; 2) augment cross-sensitization with stress; and 3) alter the effects of amphetamine on dopamine receptor expression.** Moreover, **I hypothesized that PAE will have sexually-dimorphic effects on**

both sensitization to amphetamine, cross sensitization of amphetamine and stress, and changes in dopamine receptor expression.

CHAPTER 2: Plasticity of basal HPA and dopamine systems is altered differentially in males and females by prenatal alcohol exposure and chronic variable stress.

2.1 Introduction

Alcohol consumption during pregnancy can result in fetal alcohol spectrum disorder (FASD) in the children, with a prevalence of 9/1,000 births in North America (Thanh and Jonsson, 2010). The broad range of adverse effects of FASD depend on dose, timing and duration of alcohol exposure, and include impairments in neurocognition, self-regulation, and adaptive functioning (Riley et al., 2003, Streissguth et al., 2004). Numerous 'secondary' disabilities, including substance use disorders (SUDs), have been reported following PAE (O'Connor and Paley, 2009). Consistent with these findings, rodent models of PAE show enhanced preference for alcohol (Barbier et al., 2009) and voluntary alcohol consumption in PAE offspring (Chotro et al., 2007), supporting the hypothesis that PAE results in neurobiological vulnerability to SUDs. The present study focuses on elucidating the effects of PAE on neurobiological mechanisms underlying vulnerability to SUDs.

Dysregulation of hypothalamic-pituitary-adrenal (HPA) function is typically associated with SUDs (Koob and Kreek, 2007, Lovallo, 2006). Moreover, data indicate that PAE reprograms the fetal HPA axis such that basal HPA tone is increased, and greater HPA activation and/or delayed or deficient recovery following stress are observed throughout life (Haley et al., 2006, Weinberg et al., 2008). While stress-induced HPA activation is adaptive in the short term, long-term increases in basal HPA tone and/or frequent or sustained HPA activation can lead to HPA dysregulation and alterations in physiological systems and behavior (McEwen, 2008). Thus, the ability to maintain basal hormone levels within the normal physiological range and to

turn off a stress response once initiated are as important as the ability to respond appropriately to stress initially, and the finding that all facets of HPA regulation are altered by PAE has important implications for normal offspring development.

The mesocorticolimbic DA pathway is implicated in functions such as motivation, attention, reinforcement, executive function and emotional regulation. There is significant overlap between central stress systems and DA pathways, resulting in a bi-directional interaction between stress and DA (Cabib and Puglisi-Allegra, 2012). For example, stress sensitizes healthy individuals to the rewarding effects of substance use, and can induce relapse after abstinence (Sarnyai et al., 2001), suggesting that HPA alterations provide a pathway for increased vulnerability to SUDs. Importantly, PAE produces significant alterations in DA systems by delaying development, reducing the size, structure and electrical activity of DA neurons, and reducing DA synthesis, binding, and metabolism (Cooper and Rudeen, 1988, Druse et al., 1990, Rathbun and Druse, 1985, Shetty et al., 1993, Wang et al., 2006). Together, these studies support the idea of reduced overall baseline activity of DA systems with PAE.

Both HPA and DA systems are important for resilience against, or vulnerability to, addiction (Sinha, 2009); however, effects of PAE on the interaction between HPA and DA systems have not been investigated. In the present study I examined PAE effects on HPA signaling and DA receptor expression in key brain regions where these systems intersect. Basal corticotropin releasing hormone (CRH) mRNA, as well as glucocorticoid (MR, GR), and DA receptor (DA-R) expression were measured following either no stress or chronic stress conditions. I also investigated sex differences in outcome as: 1) there are sex differences in HPA function across species, with females typically showing greater HPA responses and resistance to negative feedback compared to males (Young, 1998); 2) PAE differentially alters HPA activity

in males and females (Haley et al., 2006, Weinberg et al., 2008); and 3) sex differences are observed in SUDs (Becker and Hu, 2008). Given the links between HPA and DA systems, and the effects of PAE on these systems, I hypothesized that PAE will enhance the effects of chronic stress on basal/tonic regulation in the brain, and that sex differences will be present in the effects of both PAE and stress, with greater effects of stress on basal regulation occurring in females compared to males.

2.2 Material and methods

Breeding, experimental diets and feeding

Animal procedures were in accordance with the National Institutes of Health guidelines, and were approved by the institutional Animal Care Committee. Adult virgin female (225-260 g; n=31) and male (275-300 g; n=12) Sprague-Dawley rats were obtained from Charles River (St Constant, PQ, Canada) and put onto a 0800-1600 light schedule. Briefly, a male and female were paired, and the presence of vaginal plugs indicated day 1 of gestation (GD 1) which followed previous procedures (Hellemans et al., 2010b, Uban et al., 2010). On GD 1, females were moved to a new colony room and randomly assigned to one of three groups: 1) Prenatal alcohol exposure (PAE) - liquid ethanol diet, *ad libitum* (n = 10); 2) Pair-fed (PF) - liquid control diet; maltose dextrin isocalorically substituted for ethanol, in the amount consumed by a PAE partner (g/kg/body weight/GD), which controls for the reduced food intake typical in alcohol-consuming dams (n = 11), 3) Control (C), standard rat chow consumed *ad libitum* (n = 11). All dams had *ad libitum* access to water throughout gestation. Experimental diets provide optimal

nutrition during pregnancy (prepared by Dyets Inc. Bethlehem, PA). The current model of PAE utilized moderate levels of chronic alcohol consumption throughout gestation, which is equivalent to the first and second trimesters of human pregnancy. In the present study, pregnant females consumed an average of 0.14, 0.17, and 0.16 (g/kg body wt/day) for gestation weeks 1, 2 and 3, respectively. Previous studies that have employed the same breeding and feeding protocols found mean blood alcohol levels in dams of ~144.4 ± 31.3 mg/dl during the third week of gestation (Hellemans et al., 2010b, Uban et al., 2010). Fresh diet was presented daily at 1700-1800 hr. This schedule permits maintenance of typical corticoid circadian rhythms in PF dams, as the corticoid rhythm in animals receiving a reduced ration re-entrains to the time of feeding (Gallo and Weinberg, 1981). Experimental diets were provided from GD1- 21, at which time they were replaced with standard laboratory chow *ad libitum*.

On postnatal day 1 (PND 1), pups were weighed and litters culled to 10 (5 females, 5 males when possible (Uban et al., 2010). Dams and pups were weighed on PND 1, 8, 15 and 22, but otherwise undisturbed. Pups were weaned on PND 22 and group-housed by litter and sex. On PND 35, same-sex animals were pair-housed with a non-littermate partner from the same prenatal group. To control for potential litter effects, only one male and one female from each litter were assigned to each experimental condition of the study.

Chronic variable stress (CVS) paradigm

In adulthood (70±5 d), pairs of rats were randomly assigned to either non-CVS (no stress), or CVS, conditions. Animals in the non-CVS and CVS conditions were housed in separate colony rooms. A CVS paradigm was utilized because of its utility in mimicking the often variable, unstable, and unpredictable challenges typically experienced by individuals with a FASD. CVS

treatment consisted of ten consecutive days of exposure to variable stressors with 2 stressors per day, separated by at least 2 hours with one occurring ante meridiem (0900-1200hr) and the other post meridiem (1300-1900 hr). The CVS paradigm consisted of the following randomized stressors: 1) tail nick; 2) social isolation; 3) restraint; 4) novel cage; 5) cage tilt; 6) soiled cage; 7) platform; and 8) white noise as previously described (Hellemans et al., 2010b). The following two modifications were made: 1) a single stressor (blood collection via tail nick, weigh and cage change) was performed on days 1, 5 and 10; and 2) overnight social isolation occurred individually in small cages with corncob bedding. Blood samples were collected via tail nick, under basal conditions, on days 1, 5, and 10 of CVS, for analysis of plasma corticosterone (CORT) levels.

Brain perfusion

24 hr following the last day of CVS, all rats in both the non-CVS and CVS conditions were perfused (0900-1100 hr). Cage partners were anesthetized with isoflurane (Baxter Corporation, Mississagua, Ontario). Blood was collected from the heart for measurement of CORT, testosterone (T), estradiol (E_2) and progesterone (P_4) within 1-2 min after removal of the cage from the colony room. Rats were then perfused transcardially and extracted brains were prepped for *in situ* hybridization (ISH) and immunohistochemistry (IHC) as previously described (Williamson et al., 2010). Coronal sections were obtained throughout the entire brain (30 μm; Bregma: 4.20mm to -7.32mm (Paxinos and Watson, 2005b).

Determination of stage of estrous cycle

At the time of perfusion, stage of estrous cycle was determined as described (Uban et al., 2012). Vaginal cytology was assessed under a light microscope to identify stages of the estrus cycle.

Radioimmunoassays (RIA)

Blood samples were collected in tubes containing 100 µl EDTA, centrifuged at 3200 rpm (15 min at 4 °C), and plasma stored at 80 °C until assayed. Tail poke samples were used to measure CORT (collected between 0900-1000), and perfusion blood samples were used to measure T, P_4, E_2 (collected between 0900-1100).

CORT. Total CORT levels (bound plus free) were determined using the ImmuChemTM Corticosterone I^{125} RIA Kit (MP Biomedicals). The specificity of the antibody to CORT is 100% (minimum detectable CORT concentration was 7.7 ng/mL), and the intra-assay coefficient of variation was 7.1%.

Testosterone. ImmuChemTM Testosterone I^{125} RIA Kit (MP Biomedicals) was used. The antibody is 100% specific for testosterone and has less than 0.01% cross-reactivity with estradiol-17β, CORT and progesterone (minimum detectable testosterone concentration was 0.1 ng/mL), and the intra-assay coefficient of variation was 6.0%.

Estradiol. Siemens/DPC Estradiol Double Antibody RIA kit was used. The antiserum cross-reacts 100% for estradiol without detectable cross-reactivity with CORT or aldosterone

(minimum detectable concentration was 8 pg/mL), and the intra-assay coefficient of variation was 7.0%.

Progesterone. ImmuChemTM Progesterone RIA Kit (MP Biomedicals) was used. The antibody is 100% specific to progesterone with no detectable cross-reaction with CORT or aldosterone (minimum detectable progesterone concentration was 0.10 ng/mL), and the intra-assay coefficient of variation was 6.4%.

Preparation of brain tissue for in situ hybridization (ISH) and immunohistochemistry (IHC)

Incubations were performed at room temperature and on a shaker unless stated otherwise. In cold PBS (0.1 M phosphate buffer in 0.9% saline; pH 7.4), brain sections were transferred to a petri dish over ice, rinsed (3x 10 min), mounted onto slides, placed into an RNAse-free desiccator, and stored under vacuum overnight. Every 5th section throughout the region of interest was used per probe (n=5-8 rats per experimental group) for both ISH and IHC.

In situ hybridization

Probes and Labeling. Antisense oligonucleotide probes were used to detect mRNA levels for CRH with a standard protocol (Lan et al., 2006b). Riboprobes were used to detect mRNA levels for mineralocoid (MR) and glucocorticoid (GR) receptors (Glavas et al., 2007). Briefly, slides were removed from the desiccator, fixed in 10% Formalin (30 min), subjected to a series of washes (Lan et al., 2006b), air-dried, and stored in a desiccator overnight. 50% oligo hybridization buffer (Lan et al., 2006b) or 75% riboprobe hybridization buffer (Glavas et al., 2007) mixed with the probe (at a hybridization activity of 600,000 cpm/slide for CRH; 2,000,000

cpm/slide for GR and MR) were applied to slides, and slides covered with hybrislips (Sigma-Aldrich Canada Ltd., ON, Canada). Sections were incubated overnight (38°C) in 50% formamide humidified containers for oligonucleotide probes, or at 55°C in 75% formamide humidified containers for riboprobes. The following morning, the hybrislips were removed and post-hybridization washes comprised a series of decreasing salt concentrations (Glavas et al., 2007, Lan et al., 2006b). Sections were dehydrated in 70% EtOH (5 min) and air-dried overnight. Sections for MR and GR were placed in light tight cassettes under Kodak BioMax MR film (Eastman Kodak Co., NY, USA) and were exposed for 16 days (GR) or 9 days (MR). Film was developed with Kodak GBX developer and GBX fixer, rinsed in water and hung to dry overnight. X-ray autoradiographs were then digitized for measuring. Slides for CRH were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) and exposed for 132 days (CeA), 28 days (PVN), 104 days (mPFC,NAc) or 99 days (BNST) sealed in desiccated, light tight boxes (4°C). Slides were developed with Kodak D-19 developer (14°C) and fixed with Kodak fixer (14°C), then counterstained with Toluidine Blue, and coverslipped with Permount (Fisher Scientific Ltd., ON, Canada).

Immunohistochemistry

Fluorescent double-staining for DA receptors (D_1 and D_2) was performed on every 5th section from bregma 3.72 – 0.84 (Paxinos and Watson, 2005b) using a standard IHC protocol (Malone et al., 2008, Uban et al., 2010) with adaptations suitable for the peptides being measured and using the following antibodies: 1) primaries: mouse monoclonal Anti-Dopamine D_1 Receptor (1:450, Novas Biologicals, Littleton, CO, USA and rabbit polyclonal Anti-Dopamine D_2 Receptor (1:300, Millipore, Temecula, CA, USA); and 2) secondaries: goat anti-mouse Alexa 594 for D_1

(1:400; Invitrogen, Eugene, OR, USA) with goat anti-rabbit Alexa 488 for D_2 (1:400; Invitrogen, Eugene, OR, USA) . Briefly, a circle was traced around the tissue on the slide using a Super HT Hydrophonic Pen (Research Products International Corp.). Sections were rinsed, blocked for 2 hr, and incubated in primaries for 22 hr in a Nunc box lined with moistened Benchkote, with TBS at 4ºC. Slides were then rinsed, and incubated in secondaries for 1 hr, then rinsed, followed by a brief dip in dH_20. Slides were left to dry for 3 hours prior to being cover-slipped. These D_1 and D_2 antibodies have been previously assessed for specificity (Oda et al., 2010, Rajput et al., 2009). In the present study, controls included a series of slides involving omission of the primary and/or secondary antibodies to assess for specificity of binding using optical density measurements (Table 2.1).

Quantification of data

Densometric Analysis. Experimenters were blind to experimental conditions. ISH data from the X-ray autoradiographs were scanned and measured (Scion Image 4.0.2 software, NIH, USA). Grey level measurements were taken in the following subregions of the HPC: 1) GR: DG, CA1; 2) MR: DG, CA1-3 (Figure 2.4C). Images from CRH nuclear-emulsion dipped slides were captured under dark field on a Zeiss Axioskop2 under 5x magnification (except PVN at 10x) using Northern Eclipse Software, and measured with ImageJ. Briefly, a customized circle covering the region of interest was used for measurements (8 sections bilaterally per rat) as follows: 1) mPFC: prelimbic (PL), infralimbic (IL); 2) NAc: core, shell; 3) CeA; 4) BNST: anterior (dorsal plus ventral), posterior (fusiform plus dorsal medial); 5) PVN (Lan et al., 2006b).

Optical densities for D_1 and D_2 receptors were acquired with an Olympus FV1000 confocal microscope (20x) and analyzed with ImageJ. Optimal images were produced by customizing

Table 2.1 DA-R IHC controls.
Note: 'X' indicates inclusion of the antibody.

Control	D_1 Primary	D_1 Secondary	D_2 Primary	D_2 Secondary
1	X			
2		X		
3			X	
4				X
5	X	X		
6			X	X
7	X	X	X	
8	X		X	X
9	X	X		X
10		X	X	X
11	X			X
12		X	X	
13	X	X	X	X

imaging parameters to the brightest immunofluorescence slides for each region to avoid saturation. The middle 1.14 μm of each section was imaged, and background measurements were obtained from the glass slide adjacent to the brain section to control for differential background on slides, as D_1 and D_2 are widely distributed throughout the brain. Corrected optical density values were averaged across right and left hemispheres and across sections (i.e. 6 measures per subject per subregion).

Statistical analyses

All statistical analyses were performed with Statistica 9.0 software (StatSoft, Inc). Developmental data were analyzed using repeated-measures analyses of variance (RM-ANOVA) with prenatal group (C, PF, PAE) as the between-subjects factor; within-subjects factors were day of gestation or lactation for the dams, or postnatal day for the offspring. A separate ANOVA assessed pup body weights on the day of birth (postnatal day (PND) 1). For each probe and subregion, a RM-ANOVA was run, with prenatal group and stress (non-CVS, CVS) as between-subject factors and subregion as the within-subject factor. Data for males and females were analyzed together to evaluate sex differences for data collected prior to perfusion, but separately after perfusion to evaluate effects of estrous stage in females. Stage of estrus was used as a covariate for females and stages were grouped (proestrous/estrous, diestrus I/II). All *post-hoc* analyses utilized Newman-Keuls comparisons.

All tests of *a priori* hypotheses utilized a Bonferroni correction. I hypothesized that 1) both stress and prenatal treatment would individually and/or interactively enhance HPA sensitivity and reduce dopaminergic function, as assessed by changes in hormone levels, CRH mRNA, and glucocorticoid, cannabinoid, and dopamine receptor levels and; 2) subregions of key

brain areas (i.e. mPFC, BNST, HPC, NAc) would be differentially affected by prenatal treatment and stress, as these subregions are known to interact with brain stress systems in opposing ways.

2.3 Results

Developmental Data

Reduced body weight in PAE and PF compared to control dams during gestation but not lactation: Analysis of maternal weight throughout pregnancy revealed a significant prenatal group x gestation day interaction ($F_{6, 72}$=8.62, p<0.00; Table 2.2). PAE weighed less than C dams on GDs 7 and 21 (p's<0.02), and PF weighed less than C dams throughout pregnancy (GDs 7-21) (p's<0.03), and less than PAE dams on GD 21 (p<0.03). There were no effects of prenatal group on maternal body weights during lactation (p's>0.26).

Reduced birth weight in PAE and PF compared to control pups, but no differences during the pre-weaning period: At birth, C weighed more than PAE and PF pups (p's<0.05), and males weighed more than females (p<0.001) (main effects of prenatal group ($F_{2,28}$=4.22, p=0.02) and sex ($F_{1,28}$=24.10, p<0.001)). During the pre-weaning period there were no significant differences among prenatal groups in body weights of male ($F_{2,84}$=1.35, p=0.27) or female ($F_{2,84}$=1.09, p=0.35) pups, and both male ($F_{3,84}$=4,488.54, p<0.001) and female ($F_{3,84}$=3,411.47, p<0.001) pups from all prenatal groups gained weight throughout the preweaning period (PNDs 1-21).

Table 2.2 Dam and Offspring Body Weights (g, Mean ± SEM)

Dam Body Weight	***Prenatal Treatment***		
	Control	**Pair-fed**	**Alcohol Exposed**
Pregnant Dams (N)	11	12	12
Maternal Death/Illness (N)	0	0	0
Perinatal Death	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
Gestation Length (d)	22.9 ± 0.3	22.7 ± 0.2	23.1 ± 0.1
Litter Size	16.0 ± 0.5	15.3 ± 1.1	15.6 ± 0.8
Dam Weight (g)	---	---	---
GD1	252.7 ± 4.6	256.0 ± 2.4	251.6 ± 3.1
GD21	419.9 ± 11.3	356.0 ± 5.2[a]	375.7 ± 6.6[a]
LD1	306.7 ± 6.3	297.6 ± 3.4	300.2 ± 5.5
LD22	323.8 ± 6.1	328.1 ± 6.1	321.2 ± 5.6
Offspring Body Weight	**Control**	**Pair-fed**	**PAE**
Males			
PND1	6.7 ± 0.2	6.0 ± 0.1[b]	6.3 ± 0.2 [b]
PND22	57.6 ± 1.0	55.4 ± 1.0	55.3 ± 1.7
Females			
PND1	6.4 ± 0.2	5.8 ± 0.2 [b]	5.9 ± 0.2 [b]
PND22	54.2 ± 0.9	54.7 ± 1.2	52.3 ± 1.8

Dam and offspring body weights: [a]PF<PAE<C. [b]PAE=PF<C. Abbreviations: GD, gestation day; LD, lactation day; PND, postnatal day. These effects in the PAE group were not significant on d 5 (p's>0.39), but were highly significantly by d 10 (p's<0.001) of CVS.

Outcome Measures in Adulthood

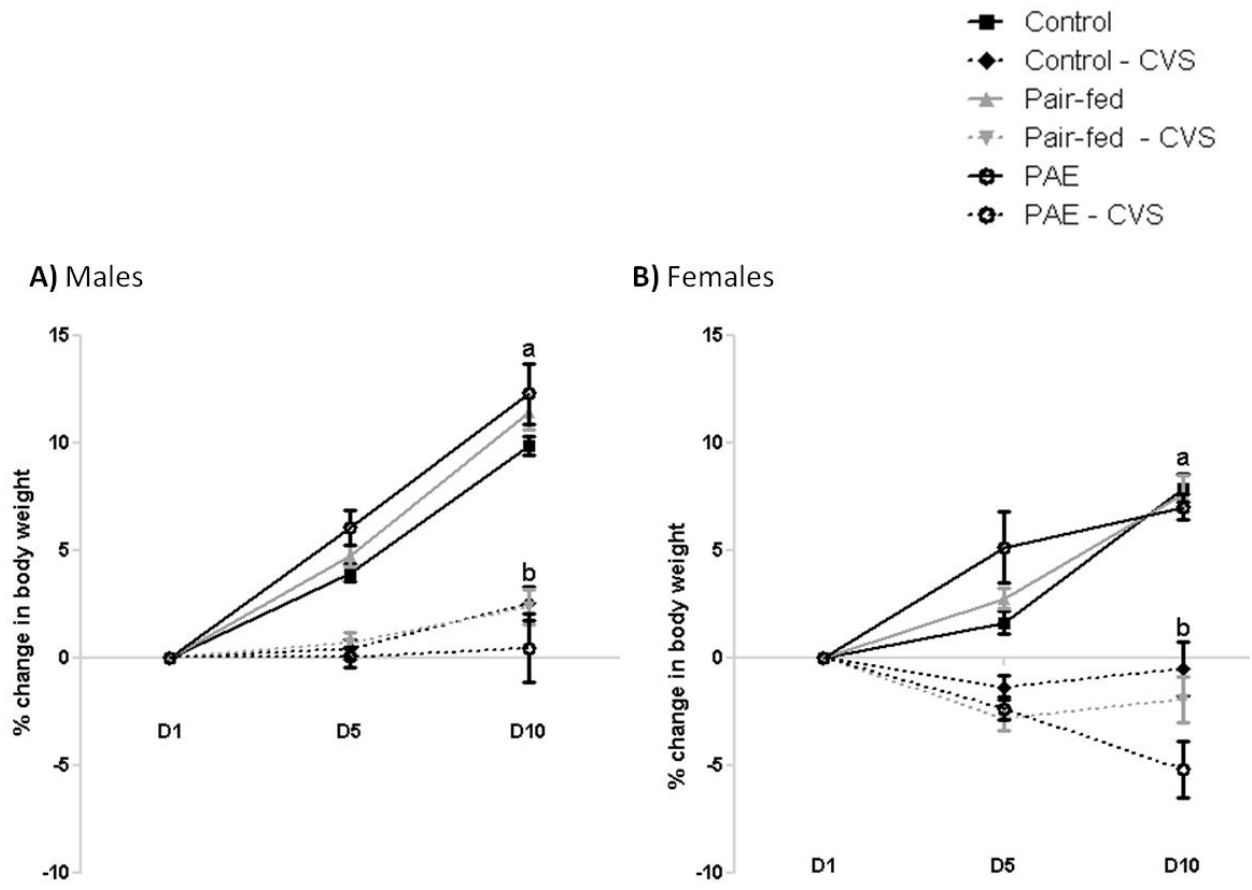
Attenuated weight gain in PAE males and weight loss in PAE females during CVS: A prenatal group x sex x stress x day RM-ANOVA on percent change in weight from D1-5, and D1-10 of CVS exposure, revealed main effects of stress ($F_{1,82}$=301.93, p<0.001), sex ($F_{1,82}$=49.31, p<0.001), and day ($F_{1,82}$=107.30, p<0.001), as well as prenatal group x stress ($F_{2,82}$=7.63, p<0.001), prenatal group x day ($F_{2,82}$=8.81, p<0.001), stress x day ($F_{1,82}$=79.87, p<0.001), and sex x day ($F_{1,82}$=9.65, p<0.01) interactions (Figure 2.1A, B). *Post-hoc* analyses revealed lower body weights during CVS in both males and females from all prenatal groups, compared to that in their respective non-CVS counterparts (p's<0.001). Overall, males exposed to CVS showed attenuated weight gain, while females exhibited weight loss (p's<0.001) on d 5 and 10 of CVS. Importantly, CVS had a significantly greater effect on PAE than PF and C animals (p's<0.01).

Hormones

CVS increased basal CORT in control, but not PAE, females, while there were no differences among groups in males: There were no significant differences in basal CORT levels among prenatal groups prior to CVS ($F_{2,42}$=0.84, p=0.43). Data were analyzed with a RM-ANOVA as percent change from D1-5 and D1-10. For males, there were no significant differences among groups in basal CORT levels over the course of CVS exposure (p's>0.41; Figure 2.2A).

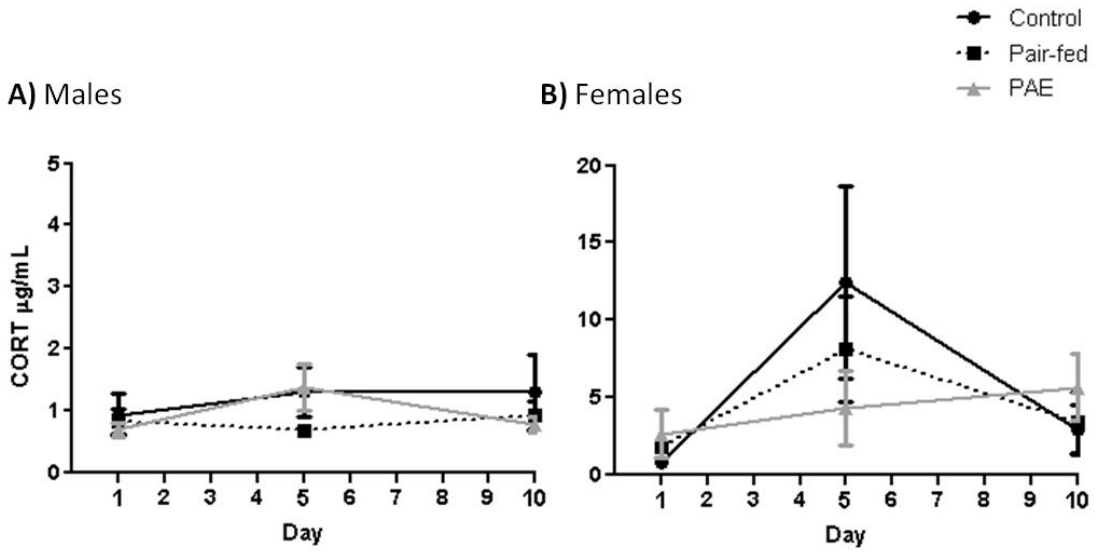
For females, a significant effect of prenatal group ($F_{2,13}$=3.56, p=0.05; 2.2B), indicated greater basal CORT levels in C compared to PAE (p=0.04), and a strong trend for greater levels in C compared to PF (p=0.056) females. Inspection of Figure 2.2 suggests that this effect of prenatal group is driven primarily by increased basal CORT from Day 1 to 5 of CVS in C females, with a return to pre-stress levels by day 10, whereas PF females show a similar, but attenuated, pattern

Figure 2.1. Effect of CVS on body weight



Effect of CVS on body weight. Offspring body weight in adulthood represented as percent change from days 1 - 5 and 1 - 10 of CVS in **A)** males and **B)** females. Mean ±SEM. Abbreviations: CVS, chronic variable stress. [a] non-CVS>CVS; [b]PAE<PF=C.

Figure 2.2. Basal CORT throughout CVS in adult offspring



Basal CORT throughout CVS in adult offspring. A) Levels of CORT under basal conditions throughout CVS are represented as total levels of CORT; **A)** males: no differences; and **B)** females: PAE<C. Note: animals subjected to CVS are represented only.

of response compared to C females, and PAE females showed a blunted response to CVS, with no change in basal CORT over the 10 days. Together, these findings suggest differential patterns of basal CORT throughout CVS in C compared to PAE, and to a lesser extent PF, females. Interestingly, basal CORT at the time of perfusion (i.e. 24 hours following the cessation of CVS) did not differ among prenatal groups or between stress conditions for either males (p's>0.22) or females (p's>0.24; data not shown).

Reduced basal levels of T in PAE males: There was a significant effect of prenatal group on T levels at the time of perfusion ($F_{2,41}$=4.36, p=0.02; C: X = 2.1 ng/mL; PF: X = 2.4 ng/mL; PAE: X = 1.2 ng/mL), but no effect of CVS (p=0.94). *Post-hoc* analysis revealed reduced levels of T in PAE compared to PF and C males (p's<0.05), while PF and C males did not differ from each other (p=0.46).

CVS reduced basal P_4, but not E_2 levels in females: As expected, P_4 levels varied as a function of estrous stage ($F_{1,38}$=5.39, p=0.05), therefore estrous stage was utilized as a covariate. There was a significant effect of stress ($F_{1,38}$=5.39, p=0.025), with reduced levels of P_4 overall following CVS (X = 44.1 ng/mL) compared to those in the non-CVS condition (X = 75.2 ng/mL). There was no effect of prenatal group (p=0.53) on P_4 levels, and no significant differences among prenatal groups or between stress conditions for E_2 levels (p's>0.10).

Effects of E_2 and P_4 on neural measures were assessed by using each hormone as a covariate for all neural measures in females. E_2 and P_4 were found to be significant covariates for the following neural measures: E_2: CRH mRNA in the PVN (p=0.02), pBNST (p=0.02), and DA-R expression in the mPFC (p=0.01); P_4: CRH mRNA levels in the IL (p=0.02). However,

overall, E_2 and also P_4 were significant covariates less frequently than stage of estrous. Importantly, significant findings for both E_2 and P_4 as covariates were virtually identical to those using stage of estrous as a covariate. Thus, stage of estrous is reported as a covariate for all measures, and not E_2 or P_4 throughout the present dissertation.

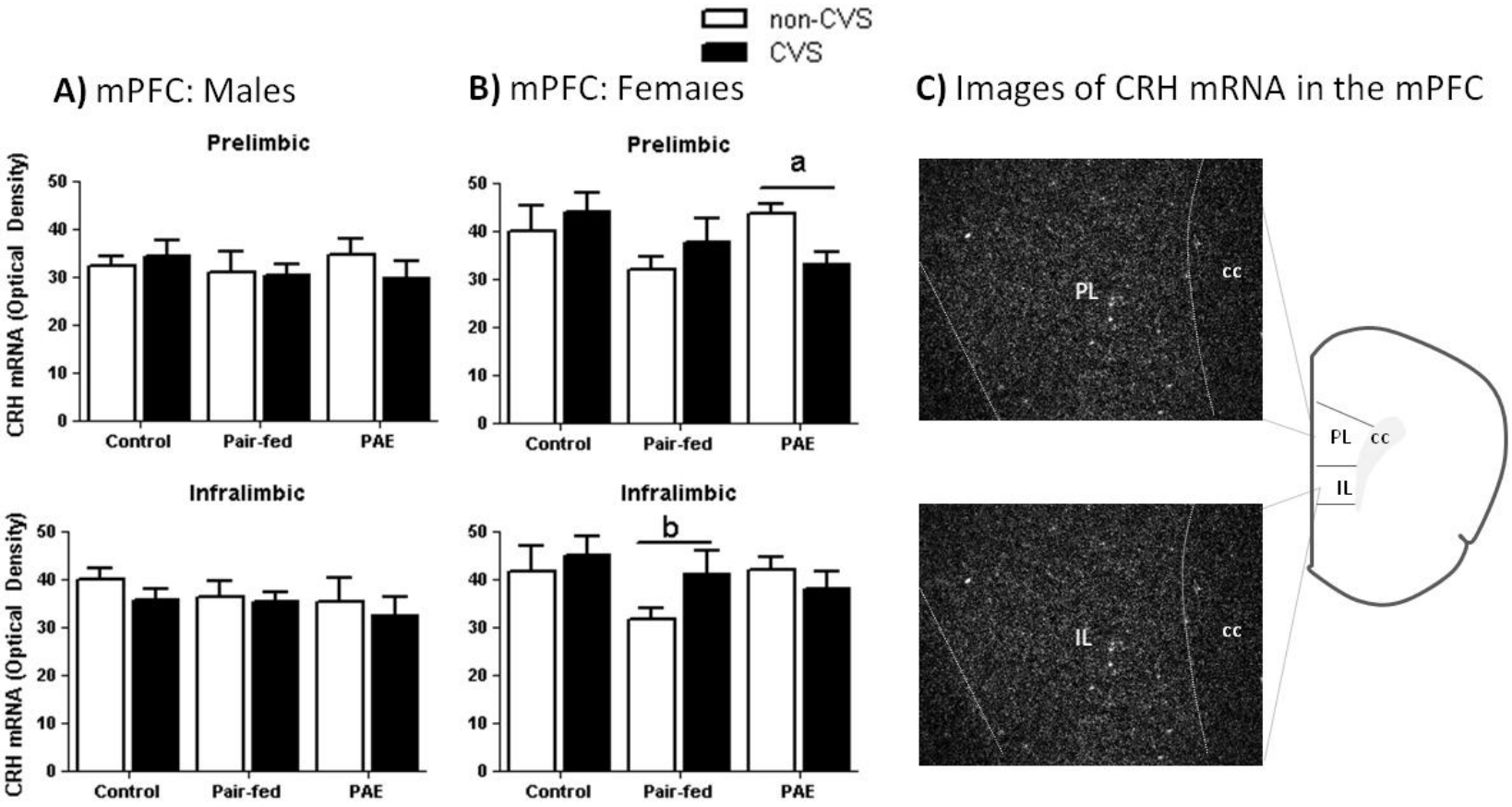
CRH mRNA signaling

Within the mPFC, CVS decreased CRH mRNA levels in the PL subregion in PAE females, but increased levels in the IL subregion in PF females: In males, CRH mRNA levels were higher in the IL compared to PL of the mPFC (main effect of subregion: $F_{1,29}=12.56$, $p<0.001$; Figure 2.3A), but there were no significant effects of prenatal group or stress on CRH mRNA levels ($p's>0.23$).

In females, while the overall ANOVA revealed no significant effects of prenatal group ($p's>0.18$), stress or subregion ($p's>0.07$), *a priori* analyses to test our hypothesis that CVS would differentially alter CRH mRNA levels in PAE compared to PF and C rats revealed that CVS decreased CRH mRNA levels in the PL of PAE females, but increased mRNA levels in the IL of PF females, compared to their control counterparts ($p's<0.0008$) (Figure 2.3B).

CVS decreased CRH mRNA levels throughout the BNST of PAE females, but only in the pBNST of control females: In males, CRH mRNA levels overall were higher in the posterior compared to the anterior BNST (main effect of subregion: $F_{1,30}=24.46$, $p<0.001$) (Figure 2.3C). There were no significant effects or interactions with prenatal group or stress ($p's>0.23$).

Figure 2.3. CRH mRNA levels



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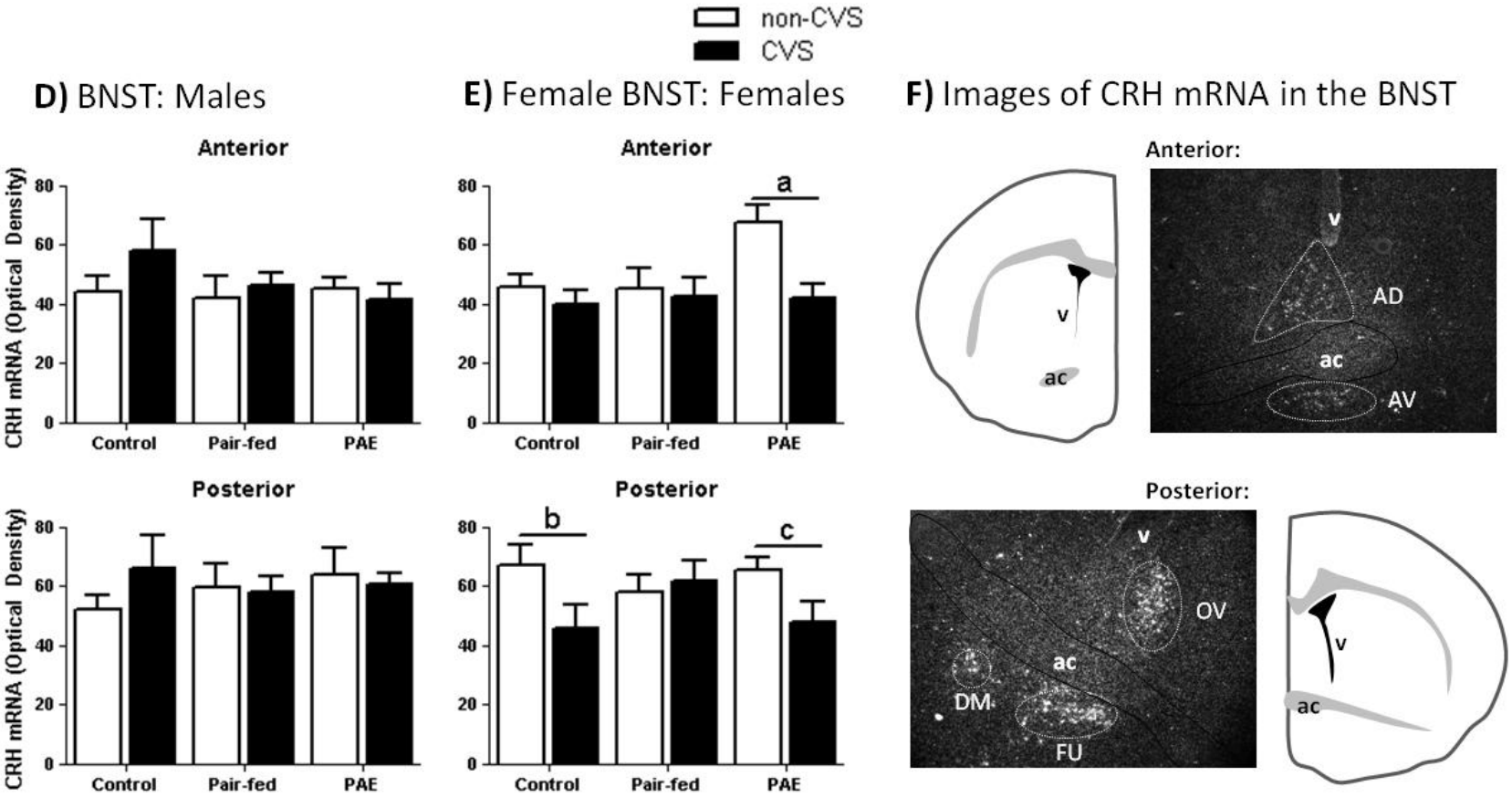


CRH mRNA levels. Mean ±SEM. Measured 24 hrs following the last day of CVS in the following regions 1) mPFC of **A)** males and **B)** females. [a]PAE: non-CVS>CVS in prelimbic (PL) subregion; [b]PF: CVS>non-CVS in infralimbic (IL) region. **C)** Images of CRH mRNA in dark field (5x) in the prelimbic (PL; top image) and infralimbic (IL; bottom image) subregions. 2) BNST of **D)** Males and **E)** females. [a]PAE: non-CVS>CVS in anterior BNST; [b]C: CVS>non-CVS in posterior BNST; [c]PAE: CVS>non-CVS in posterior BNST. **F)** Images of CRH mRNA in dark field (10x) in the anterior (top image) and posterior (bottom image) BNST. Abbreviations: anterior dorsal (AD); anterior ventral (AV); anterior commisure (ac); corpus callosum (cc); dorsalmedial (DM); fusiform (FU); oval (OV); ventricle (v).

In females, CVS decreased CRH mRNA levels in the BNST overall (main effect of stress: $F_{1,38}$=7.10, p=0.01), and there was a trend for a prenatal group x subregion interaction ($F_{2,38}$=2.89, p=0.067) (Figure 2.3D). *A priori* analyses revealed that CVS reduced CRH mRNA levels in both the anterior and posterior BNST in PAE females, but only in the pBNST of C females (p's<0.0008).

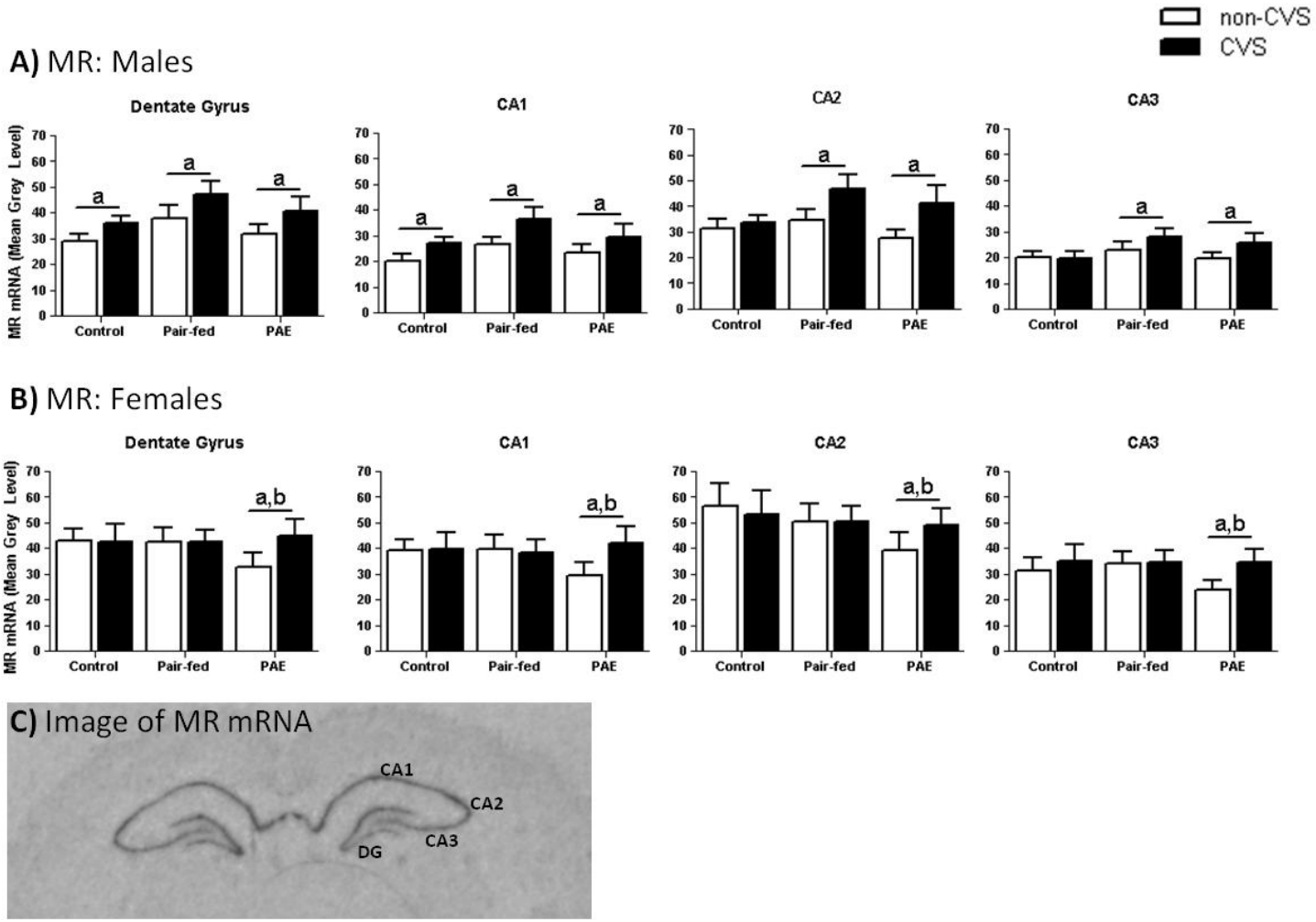
No significant group differences in CRH mRNA levels in the NAc shell, CeA, or PVN: There were no significant differences in CRH mRNA expression in the NAc, CeA and PVN among prenatal groups or following stress in either males (p's>0.25), or females (p's>0.16; data not shown).

MR and GR mRNA levels the hippocampus

CVS increased MR mRNA levels throughout the HPC in PAE males and females: In males, there were significant effects of stress ($F_{1,123}$=5.44, p=0.02) and subregion ($F_{3,\ 123}$=138.36, p<0.001), a stress x subregion interaction ($F_{3,123}$=4.86, p=0.003), and a statistical trend for a prenatal group x stress x subregion interaction ($F_{6,123}$=2.08, p=0.06) (Figure 2.4A). *A priori* analyses revealed that CVS increased MR mRNA levels throughout the HPC in PAE and PF males, but only in the DG and CA1 of C males (p's<0.0125).

In females, stage of estrus was a significant covariate ($F_{1,114}$=4.74, p=0.03), such that MR mRNA levels were increased throughout the HPC during proestrus/estrus compared to diestrus. As well, *a priori* analyses revealed that under non-CVS conditions, MR mRNA levels were lower in PAE than in PF and C females, and CVS then increased MR mRNA levels in PAE but

Figure 2.4. mRNA levels in the HPC



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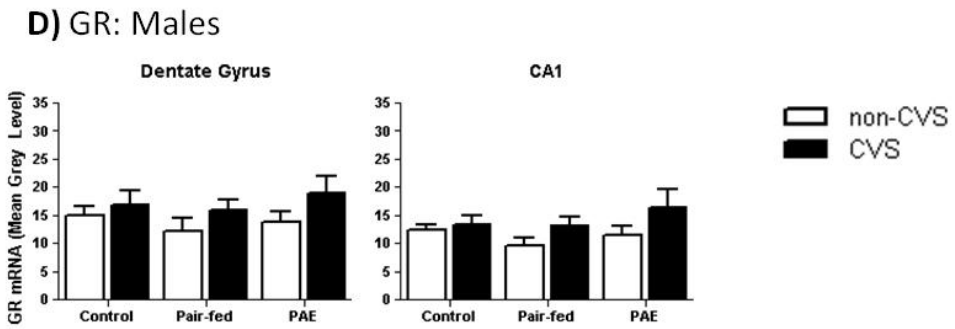
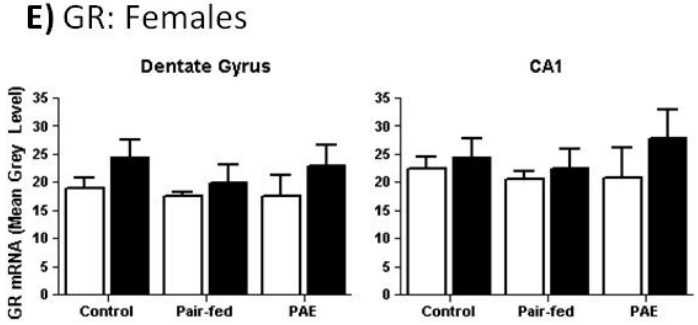




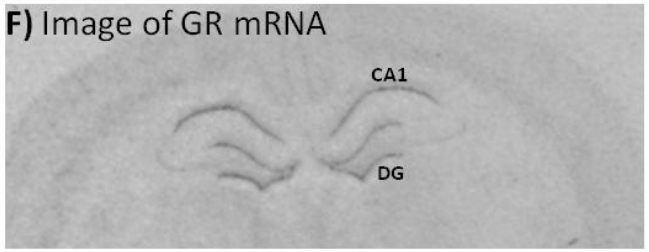


Figure 2.4. mRNA levels in the HPC. Mean ±SEM. Measured 24 hrs following last day of CVS. MR mRNA in **A)** males and **B)** females. [a]CVS>non-CVS; [b]PAE<C=PF. **C)** Image of MR mRNA expression on film with the dentate gyrus (DG), CA1, CA2 and CA3 subregions. GR mRNA in **D)** males and **E)** females. In males, there was a main effect of CVS overall: CVS>non-CVS. **F)** Image of GR mRNA expression on film with the dentate gyrus (DG) and CA1 subregions.

not PF or C females (p's<0.0125) (Figure 2.4B). There were no other significant main effects or interactions with prenatal group or following stress in females (p's>0.21).

CVS increased GR mRNA levels in the HPC of males, but not females overall: In males, there was a statistical trend for an effect of CVS on GR mRNA levels ($F_{1,38}$=3.72, p=0.06), with higher GR mRNA levels following CVS overall (non-CVS: $\bar{x}$ = 12.48; CVS: $\bar{x}$ = 15.81).

In females there was a statistical trend for stage of estrus cycle as a covariate ($F_{1,35}$=3.41, p=0.07), with increased GR mRNA during proestrus/estrus, and estrus stage was utilized as a covariate in this analysis. However, there were no significant effects or interactions with prenatal group or stress among females (p's>0.12) (non-CVS: $\bar{x}$ = 19.91; CVS: $\bar{x}$ = 23.76; C: $\bar{x}$ = 22.15; PF: $\bar{x}$ = 20.30; PAE: $\bar{x}$ = 23.16).

DA receptor expression

No significant prenatal group differences in DA-R expression throughout the mPFC: In males, there was an interaction between DA-R subtype (i.e. D_1 or D_2) and subregion ($F_{(1,41)}$=5.32, p=0.02). There was equal expression of D_1 in the PL and IL (p=0.86), but greater expression of D_2 in the PL compared to IL (p<0.01); data not shown). There were no other significant main effects or interactions (p's>0.09).

In females, there was a significant effect of DA-R subtype ($F_{1,41}$=10.13, p<0.003), with greater expression of D_1 than D_2 overall (p's<0.001). There were no significant effects of prenatal group or stress (p's>0.16; data not shown).

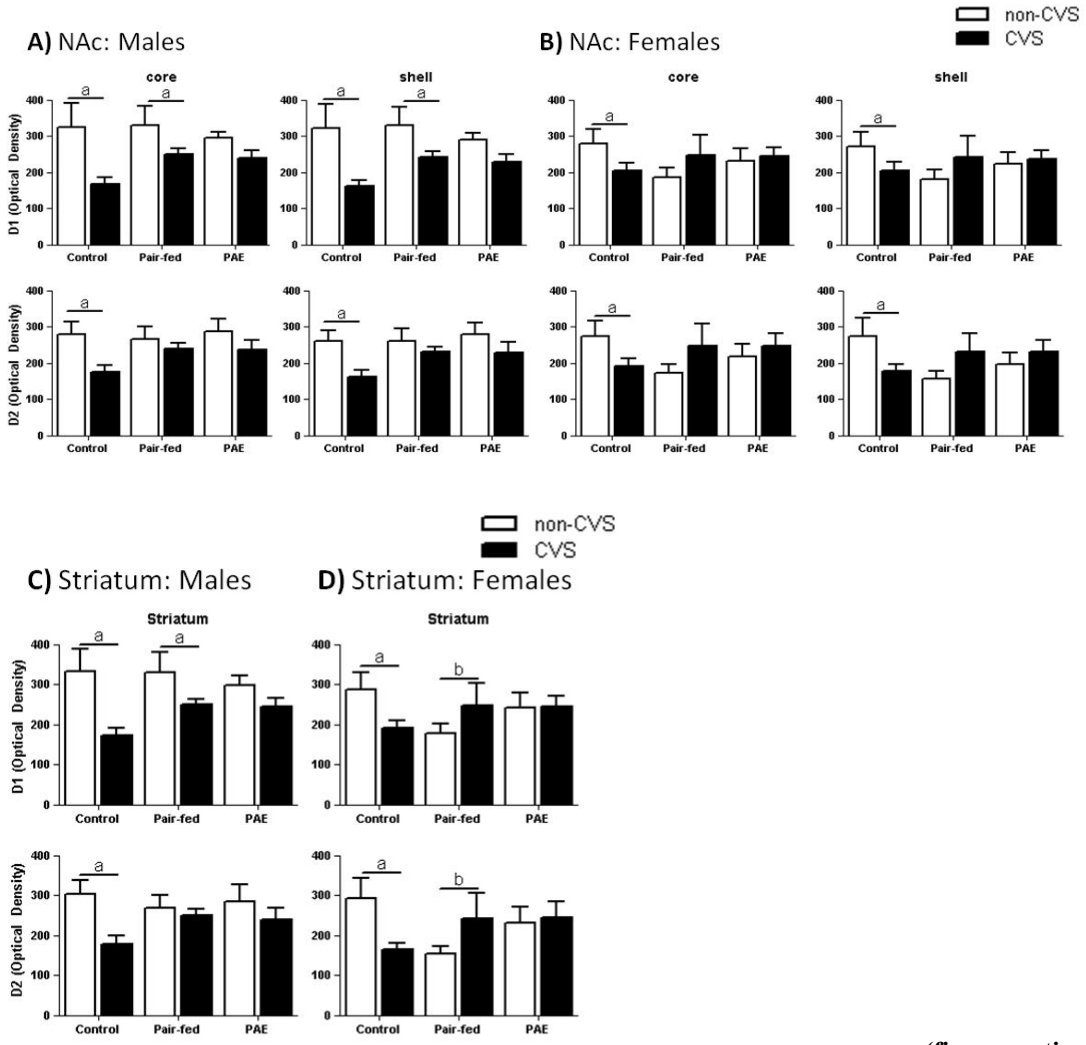
CVS reduced DA-R expression in the NAc in control but not PAE rats: In males, there were significant effects of stress ($F_{1,41}$=10.95, p=0.001), DA-R subtype ($F_{1,41}$=4.78, p=0.03) and NAc subregion (i.e. core, shell) ($F_{1,41}$=25.83, p=0.001), and a significant DA-R subtype x subregion interaction ($F_{1,41}$=2.52, p=0.002). *Post-hoc* analyses revealed that overall, there was greater D_1 compared to D_2 expression (p's<0.001), and greater DA-R expression in the core compared to the shell (p's<0.001) (Figure 2.5A). As well, *a priori* analyses revealed that CVS reduced DA-R expression in the NAc core and shell in C males (p's<0.0006), reduced D_1, but not D_2, expression overall in PF males (p's<0.0006), and had no effect on DA-R expression in PAE males (p>0.26)

In females, there was a significant effect of subregion ($F_{1,41}$=14.25, p<0.001), with greater expression of both D_1 and D_2 overall in the core compared to the shell (Figure 2.5B). In addition, *a priori* analyses revealed that CVS reduced DA-R expression in both the core and shell in C females (p's<0.0006), but had no effect in PF and PAE females (p's>0.50).

CVS reduced DA-R expression in the striatum in control but not in PAE rats: In males, CVS reduced DA-R expression in the striatum overall (main effect of stress; $F_{1,41}$=10.85, p=0.002) (Figure 2.5C). Moreover, *a priori* analyses revealed differential effects of CVS depending on prenatal treatment: CVS reduced DA-R expression in C males, reduced D_1 expression in PF males (p's<0.016), and had no effect on DA-R expression in PAE males (p's>0.25).

In females, there was a statistical trend for an interaction between prenatal group and stress ($F_{2,41}$=2.74, p=0.07) (Figure 2.5D). *A priori* analyses revealed that CVS reduced DA-R expression in C females, increased DA-R expression in PF females (p's<0.016), and had no effect in PAE females (p's>0.43).

Figure 2.5. Optical densities of DA-R expression



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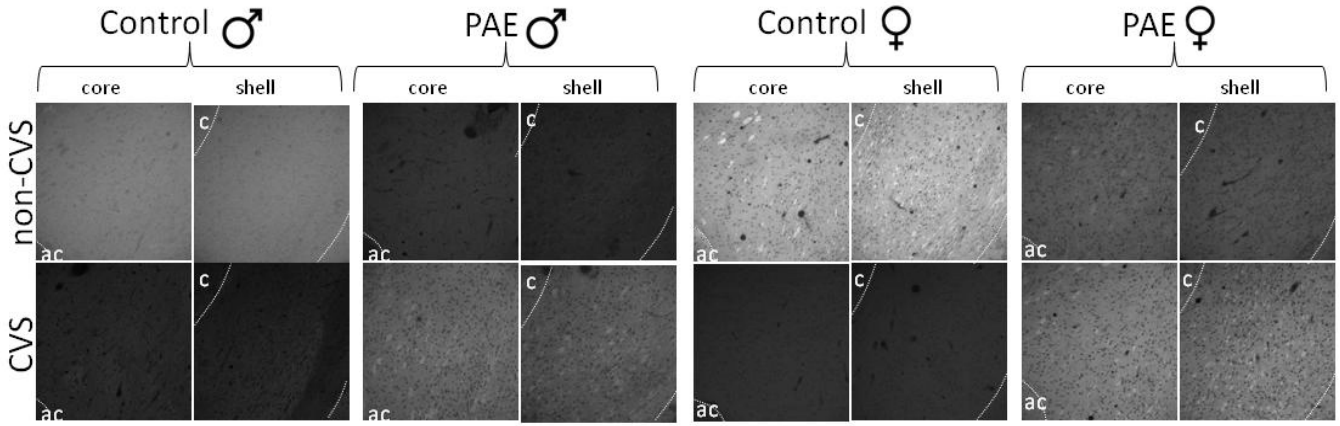
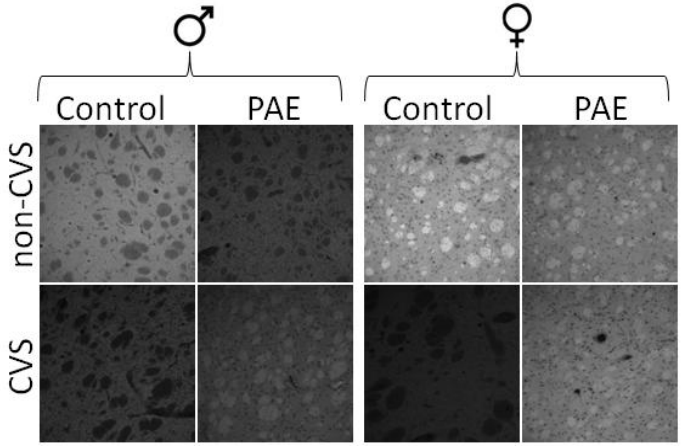
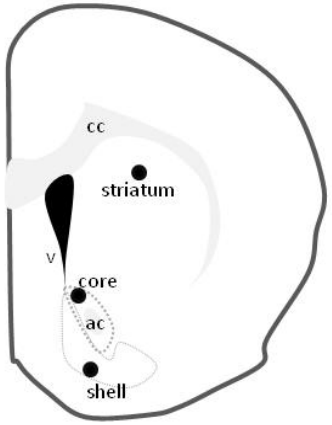
E) NAc



F) Striatum





Optical densities of DA-R expression. Mean ±SEM. D_1 & D_2 expression was measured 24 hrs following last day of CVS. The core and shell subregions of the NAc in **A)** males and **B)** females. [a]non-CVS>CVS in C and PF, but not PAE males and females. The striatum in **C)** males and **D)** females. Mean ±SEM. [a]non-CVS>CVS in C but not PAE males and females; [b]CVS>non-CVS in PF females. Images of D_1 expression (20x) under non-CVS (top rows) and CVS (bottom rows) conditions in Control and PAE males (left) and females (right) in the **E)** NAc core and shell and **F)** striatum. Abbreviations: anterior commisure (ac); corpus callosum (cc); ventricle (v).

Correlations between DA-R densities and Hormones:

Testosterone: Higher T levels were positively correlated with greater D_2 densities in the striatum following CVS in PAE males only ($r(8)=.75$ $p<0.03$), and there was a similar trend for D_1 ($r(8)=.69$, $p=0.056$). There were no other correlations between T levels and DA-R densities ($p's>0.10$).

Estradiol: There was a significant positive correlation between E_2 and D_1 levels in PAE females following CVS in the PL ($r(8)=.66$, $p=0.035$) and IL regions ($r(8)=.68$, $p=0.028$), and a similar correlation for PF females under non-CVS conditions for both D_1 (PL: ($r(6)=.76$, $p=0.029$; IL: ($r(6)=.74$, $p=0.032$) and D_2 (PL: ($r(6)=.81$, $p=0.013$; IL: ($r(6)=.79$, $p=0.018$). There were no other significant correlations between E_2 levels and DA-R densities ($p's>0.19$).

Thus dysregulation in dopamine systems of PAE subjects may be influenced by alterations in gonadal hormones, evidenced by the presence of significant positive relationships between hormone levels and DA-R expression, with no such associations present in Control subjects.

Basal CORT: In PAE females under non-CVS conditions only, basal CORT at the time of perfusion was significantly positively correlated with D_2 expression in the IL ($r(4)=.82$, $p=0.044$) and NAc shell ($r(4)=.82$, $p=0.044$), with a similar trend in the PL ($r(4)=.80$, $p=0.052$) and NAc core ($r(4)=.79$, $p=0.056$). A similar positive correlation was observed under non-CVS conditions among PF females in the mPFC with both D_1 (PL: ($r(6)=.85$, $p=0.006$; IL: ($r(6)=.84$, $p=0.008$)

and D_2 (PL: (r(6)=.91, p=0.001; IL: (r (6)=.91, p=0.001). There were no other significant correlations between basal CORT levels and DA-R densities (p's>0.18).

In males, there were no significant correlations between basal CORT levels and DA-R expression (p's>0.13).

2.4 Discussion

CVS unmasked alterations in central HPA circuitry and a loss in the normal plasticity of dopamine systems in PAE compared to control animals under basal conditions (see Table 2.2 for summary). Specifically, PAE increased sensitivity to stress, and altered interactions between HPA and DA systems, as shown by differential effects of CVS on: 1) body weights of PAE compared to control females; 2) patterns of basal CORT over the course of CVS in PAE compared to control females; 3) downregulation of basal CRH mRNA levels in the mPFC and BNST of PAE compared to control females; 4) upregulation of basal MR mRNA levels throughout the hippocampus of PAE males and females compared to their control counterparts; and 5) downregulation of basal DA-R expression in control, but not PAE, animals. Overall, these results extend our understanding of the sexually-dimorphic effects of PAE on basal HPA regulation, and show, for the first time, that plasticity of basal HPA *and* DA systems is altered differentially in males and females by PAE and CVS. Further, dysregulation of DA systems by PAE may be influenced by alterations in gonadal hormones, evidenced by the presence of significant positive relationships between hormone levels and DA-R expression in PAE but not Control subjects.

Table 2.3 Summary of neurobiological effects of chronic variable stress:

		Males			Females		
		Control	**Pair-fed**	**PAE**	**Control**	**Pair-fed**	**PAE**
mPFC	**PL**	--	--	--	--	--	↑ CRH mRNA
	IL	--	--	--	--	↓ CRH mRNA	--
BNST	**aBNST**	--	--	--	--	--	↓ CRH mRNA
	pBNST	--	--	--	↓ CRH mRNA	--	↓ CRH mRNA
HPC	DG	↑MR & GR mRNA	↑MR & GR mRNA	↑MR & GR mRNA	--	--	↑MR mRNA
	CA1	↑MR & GR mRNA	↑MR & GR mRNA	↑MR & GR mRNA	--	--	↑MR mRNA
	CA2	--	↑MR mRNA	↑MR mRNA	--	--	↑MR mRNA
	CA3	--	↑MR mRNA	↑MR mRNA	--	--	↑MR mRNA
NAc	core	↓ D_1 & D_2	↓ D_1	--	↓ D_1 & D_2	--	--
	shell	↓ D_1 & D_2	↓ D_1	--	↓ D_1 & D_2	--	--
Striatum		↓ D_1 & D_2	↓ D_1	--	↓ D_1 & D_2	↑ D_1 & D_2	--

Summary of basal changes in central measures 24 hrs following the last day of CVS (or non-CVS) in the: 1) prelimbic (PL) and infralimbic (IL) subregions of the mPFC; 2) anterior (aBNST) and posterior (pBNST) subregions of the BNST; 3) in the dentate gyrus (DG), CA1, CA2 and CA3 subregions of the HPC; 4) the core and shell of the NAc; and 5) in the striatum. Abbreviations: corticotropin releasing hormone (CRH); mineralcorticoid receptor (MR); glucocorticoid receptor (GR); dopamine receptor type I (D_1) and type II (D_2). (all p's<0.05)

Body weight is influenced by PAE and CVS

As expected, both PAE and PF pups exhibited reduced birth weights. However, catch up growth was observed throughout the preweaning period. These results are consistent with past studies (Hellemans et al., 2010b, Uban et al., 2010), showing that our model of PAE produces mild-moderate effects on fetal growth, and is a model of alcohol related neurodevelopmental disorder (ARND) rather than FAS. Previous studies using the same protocol found moderate blood alcohol levels in dams (mean ~145 mg/dl) during the third week of gestation (Hellemans et al., 2010b, Uban et al., 2010). Importantly, CVS in adulthood attenuated weight gain in PAE males and weight loss in PAE females compared to controls. These results support the hypothesis that PAE produces enhanced sensitivity to CVS in both sexes.

Endocrine regulation is altered by PAE

Prenatal treatment altered the pattern of basal CORT activity during CVS in females. Control females showed an elevation of basal CORT levels midway through CVS treatment, and a return to baseline levels by the end of CVS. This profile of basal CORT levels was attenuated in PF females and absent in PAE females, suggesting that PAE blunts the normal basal CORT response to CVS. Interestingly, basal CORT levels were unaltered in males on the day of termination. Following a 24 hour washout period, central dysregulation in stress systems was observed in the absence of changes in basal hormone levels in animals subjected to CVS, and this disconnect was greater in PAE than in control animals. This finding is important, as a disconnect between central and peripheral measures is known to be associated with increased

vulnerability to a range of mental health problems (de Kloet et al., 2005). For example, the extrahypothalamic regions are more remote from the hypothalamus, and therefore alterations in basal MR/GR or CRH mRNA expression can contribute to vulnerability to mental health problems in the absence of basal hypothalamic alterations (de Kloet et al., 2005). Alterations within the extrahypothalamic regions may be an antecedent for alterations in cognition, behavior and affect associated with mental health problems.

Reduced basal testosterone levels were observed in PAE compared to control and PF males, supporting past findings that PAE reduces sensitivity of the HPA axis to the regulatory effects of testosterone (Lan et al., 2009) and may result in HPG dysregulation. Testosterone typically plays an inhibitory role on HPA activity (Viau, 2002). In PAE males, low testosterone levels could diminish the capacity to regulate HPA activity and contribute to the HPA hyperresponsiveness in PAE animals that is typically observed.

In females we found basal progesterone levels were reduced following CVS, but no significant effect on estradiol levels. Progesterone functions as a glucocorticoid antagonist on the HPA axis (reviewed in (Kudielka and Kirschbaum, 2005). It is possible that decreased basal progesterone levels results in reduced antagonism of HPA activity, and provides one mechanism through which CVS increases overall vulnerability to subsequent stressors in females.

Overall, basal hormone levels were measured in the current study; therefore we cannot determine how gonadal hormone levels would be altered under a stress-activated state. Past research has shown that stress-induced T levels were *elevated* following CVS exposure compared to that in animals from the non-CVS condition (Hellemans et al., 2008), while our results show *reduced* basal T levels following CVS. Similarly, stress-induced E_2 levels were *reduced* following CVS in PAE and PF females (Hellemans et al., 2008), while our results found

no differences in basal E_2 levels following CVS. Stress-induced P levels were *elevated* following CVS in PAE compared to PF and C females (Hellemans et al., 2008), yet the present study found *reduced* basal P levels following CVS. Together, the divergent effects of CVS on gonadal hormones under basal compared to stress-activated states provides a more complete picture of whole system changes of the HPG axis following CVS. These data suggest that prior stress exposure may have differential effects on basal versus stress-activated levels of gonadal hormones, and which may actually be altered in opposing directions.

PAE alters basal HPA signaling in females, but not males, in a region specific manner

There were no pre-existing differences in baseline CRH mRNA expression between PAE and control females. However, CVS revealed basal CRH mRNA alterations in the mPFC and BNST of PAE and PF compared to control females, which suggest enhanced sensitivity to CVS. Within the mPFC, the PL subregion inhibits, while the IL subregion potentiates, activation of brain stress systems (Sullivan and Gratton, 2002). Similarly, subregions within the BNST have opposing effects: the aBNST increases, while the pBNST decreases, CRH mRNA expression in the PVN of the hypothalamus (Herman et al., 1994). Here, I found a *decrease* in CRH mRNA levels following CVS in the PL of PAE females, but an *increase* following CVS in the IL subregion of PF females. Together, these data suggest that CVS increases HPA drive within the mPFC in both PAE and PF females, via different mechanisms, where inhibitory drive is decreased in PAE females, and excitatory drive is enhanced in PF females. Within the BNST, CRH mRNA levels were reduced in both subregions of the BNST in PAE females, but only in the pBNST of control females. These findings suggest that CVS decreased inhibitory regulation

in control females, which may account for the increased basal CORT levels on day 5 of CVS. In contrast, both inhibitory and excitatory drive are altered in PAE females, reflecting broader effects of CVS in PAE than controls, and possibly contributing to the overall blunting of the basal CORT response to CVS.

Interestingly, the effects of CVS and PAE in the mPFC and BNST were sexually-dimorphic, as there were no effects of CVS on CRH mRNA among males. Thus, prenatal treatments such as PAE or pair-feeding have a greater impact on sensitivity to stress and basal CRH activity or regulation in females compared to males. Consistent with this, prenatal stress caused HPA hyperactivity selectively in adult females, suggesting sex differences in the sensitivity of the developing brain to stress hormones (Weinstock et al., 1992). By analogy, I suggest that prenatal exposure to alcohol, which has both direct and indirect (via maternal HPA activation) effects on the developing fetal HPA axis, may selectively alter CRH regulation in adult female offspring. However it is important to note that it in the present study basal HPA regulation was altered, whereas alterations in HPA responsiveness to stressors were found in past data using prenatal stress (Weinstock et al., 1992).

I found that CVS increased MR mRNA levels throughout the hippocampus of PAE animals, whereas GR mRNA levels were upregulated following CVS in males, but not females, across all prenatal groups. GRs play a role in suppression of stress-induced HPA activity, whereas MRs play a permissive or tonic role in regulating basal HPA activity (De Kloet et al., 1998, Sapolsky et al., 2000). Shifts in the balance between MRs and GRs alter stress system activity and decrease the ability to maintain homeostasis. The finding of MR mRNA upregulation under basal conditions in PAE offspring following CVS suggests an increase in basal HPA tone. Furthermore the overall upregulation in GR mRNA levels in males, but not

females, suggests that males are more sensitive than females to the effects of CVS on the hippocampus.

Past studies utilizing either a 10 or 21 day repeated restraint paradigm (Park et al., 2011, Wright et al., 2006) (Zhou et al., 2008) found *decreased* GR, but unaltered MR protein expression or mRNA levels following stress. A decrease in GR may reflect a compensatory mechanism to decrease sensitivity or responsiveness to subsequent stressors. Discrepancies between results of these past studies and those of the present study may be due to the: 1) type of stressor (repeated exposure to the same stressor vs variable stressors); 2) stressor intensity (daily restraint of 5-6 hr vs exposure to moderate stressors for shorter durations); and/or 3) examination of mRNA levels under stress-activated versus basal conditions. It is possible that more prolonged homotypic stress regimens result in the downregulation of GR, while shorter heterotypic stress regimens result in upregulation of GR and more selectively of MR. Interestingly, while the interaction between PAE and stress resulted in MR mRNA upregulation in the hippocampi of both males and females, it appears that in PAE females this effect was primarily driven by reduced basal MR mRNA levels, suggesting that the underlying mechanisms of this effect are sexually-dimorphic.

Stress reduced basal DA-R expression in control but not PAE animals

Exposure to CVS reduced DA-R expression in the striatum and NAc in both control males and females, but not in PAE subjects. While acute stress enhances DA neuron activity, chronic stress may attenuate this effect (Valenti et al., 2012), possibly by initially increasing and

then decreasing, tonic DA activity in the NAc (Cabib and Puglisi-Allegra, 2012). In the present study, it is possible that the typical stress-enhanced DA release became increasingly blunted over time by CVS, resulting in reduced tonic activity and stress-induced levels of DA in controls. As chronic stress decreases DA levels in the long-term, DA-Rs may eventually downregulate to match the reduced tonic DA levels. The downregulation of DA-Rs following CVS observed in controls may result, in part, from an overall downregulation in activity of DA systems.

Additionally, our results suggest that DA regulation in the NAc and striatum is more malleable by CVS than DA regulation in the mPFC. The NAc and striatum are regions of importance for DA function in relation to reinforcement and motivational neurocircuitries. The mPFC directly influences tonic DA in the NAc as it evaluates the stressor (Cabib and Puglisi-Allegra, 2012); therefore alterations in DA-R expression may be revealed in the NAc prior to alterations occurring in the mPFC. Our results are the first to demonstrate that CVS dowregulates basal DA-R expression in both sexes and that PAE attenuates the effects of CVS on DA systems in brain regions highly relevant to the etiology of SUDs.

PAE rats show increased HPA responsivity to alcohol and morphine challenges compared to control animals (Taylor et al., 1986), and enhanced stress-induced alcohol consumption (Nelson et al., 1983). The present data support and extend these findings, indicating that PAE attenuates plasticity of DA systems following CVS. Together, these findings suggest that PAE reduces meta-plasticity of DA systems, where dopaminergic responses are not as finely calibrated to a range of stimuli, including an array of pharmacological agents, and in the present study, stress. As a result, PAE subjects may show exaggerated or blunted plasticity of DA systems compared to control subjects.

In the present study, we found a significant relationship between gonadal hormone levels and DA-R expression in PAE but not control subjects, and these relationships differed depending on whether or not animals had previous exposure to CVS. These findings suggest that gonadal hormones may play a greater role in HPA-DA interactions in PAE than in control animals.

Pair-feeding is an experimental treatment in itself

A number of effects of pair-feeding were observed in the present study. It is important to note that although pair-feeding controls for the reduced nutritional intake of the PAE dams, it is actually a treatment. Although alcohol consuming dams eat *ad libitum*, they typically reduce their intake below what would occur if given the same diet without alcohol. Because the amount of diet presented to PF dams is yoked to that of PAE, PF dams are effectively on a "meal feeding" schedule. They eat less than they would eat *ad libitum*, and consume their entire ration within a few hours, remaining deprived until the next feeding. It is likely that PF dams experience some level of stress as a result of potential hunger that accompanies reduced food intake, and in turn, their offspring may experience some level of prenatal stress. Thus, it is not entirely surprising that some effects of pair-feeding on central HPA systems and DA systems are observed in the present study and are unique from effects observed in PAE subjects.

Conclusions

The present data demonstrate that PAE produces long-lasting alterations in both HPA and DA systems, as well as in HPA-DA interactions. Typically, PAE results in increased

responsiveness to stressors. However, if the system is challenged, HPA dysregulation under basal conditions may be revealed (Glavas et al., 2007), indicating enhanced HPA drive. The significance of the present results is the finding of pronounced alterations in central HPA regulation in the face of similar basal CORT levels. It is possible that these changes in central regulation of basal HPA activity may underlie the increased stress responsiveness that is consistently observed in PAE animals and in FASD children, as well as enhanced vulnerability to a range of mental health disorders (de Kloet et al., 2005) including substance use disorders. These findings enhance our understanding of PAE effects on the cross-talk between HPA and DA systems, and provide insight into possible mechanisms underlying mental health problems that are related to stress and DA signaling, including SUDs, which have a higher incidence among FASD individuals (O'Connor and Paley, 2009). Moreover, optimal dopaminergic function is required for executive function, cognition, and emotional regulation, (Goto et al., 2007), all of which are altered by PAE (Mattson et al., 2011, Schneider et al., 2011). An understanding of how PAE alters the neurobiological mechanisms implicated in mental health problems is vitally important for the development of specialized prevention, intervention and treatment for this unique population.

CHAPTER 3: Prenatal alcohol exposure enhances sensitization to repeated amphetamine exposure and cross-sensitization with acute stress.

3.1 Introduction

Alcohol consumption during pregnancy can result in fetal alcohol spectrum disorders (FASD) in the children, with a prevalence of 9/1,000 births in North America (Sampson et al., 1997, Thanh and Jonsson, 2010). The broad range of adverse effects of FASD depend on dose, timing and duration of alcohol exposure, and include impairments in cognition, self-regulation, and adaptive functioning (Coles et al., 2009, Rasmussen, 2005, Riley et al., 2003, Spohr et al., 2007, Streissguth and O'Malley, 2000). Numerous secondary disabilities, including mental health problems such as depression and substance use disorders (SUDs), have been reported following prenatal alcohol exposure (PAE) (Alati et al., 2008, Baer et al., 2003, O'Connor and Paley, 2009). Consistent with these findings, rodent models of PAE show increased vulnerability to depressive-/anxiety-like behavior (Hellemans et al., 2010b) and enhanced preference for, and voluntary consumption of, alcohol in PAE offspring (Barbier et al., 2009, Chotro et al., 2007), supporting the hypothesis that PAE results in neurobiological vulnerability to mental health and SUDs. The present study focuses on elucidating the effects of PAE on neurobiological mechanisms important for the development of SUDs.

Dysregulation of dopamine systems is implicated in enhanced vulnerability to SUDs (Di Chiara and Bassareo, 2007, Everitt et al., 2008, Le Moal, 2009, Sinha, 2008, Sweitzer et al., 2012, Volkow et al., 2011). For example, hypersensitivity of underlying dopaminergic neural

circuitry in rats can be observed following repeated exposure to stimulants, and is referred to as behavioral sensitization (Robinson and Berridge, 1993). Indeed, sensitization of dopamine systems is positively correlated with vulnerability to developing SUDs, and to subsequent relapse following prolonged abstinence (Piazza et al., 1990, Robinson and Berridge, 2003). Thus, a paradigm involving repeated exposure to stimulants such as amphetamine (AMPH) provides a behavioral measure indicative of neurobiological vulnerability to SUDs, particularly pertaining to dopamine systems.

PAE produces marked alterations in dopamine systems, including reduced dopamine cell body size, dendritic growth, neuronal electrical activity, synthesis, receptor binding sites, uptake sites, and metabolites (Blanchard et al., 1993, Shen et al., 2007, Shen et al., 1999, Shetty et al., 1993, Spear, 1996, Wang et al., 2006). These studies demonstrate overall reduced tonic activity of dopamine systems following PAE, which may contribute to neurobiological mechanisms underlying vulnerability to SUDs in this population. Furthermore, I have shown previously that although baseline dopamine receptor (DA-R) expression was not altered by PAE in the nucleus accumbens (NAc) or striatum, exposure to stress resulted in downregulation of basal DA-R expression in control but not PAE subjects, suggesting that PAE attenuates the typical effects of chronic stress on basal expression of DA-Rs in both males and females (Uban et al., submitted-a). Similarly, it was shown that the normal drug-induced increase in dopamine content in the NAc is attenuated in PAE rats (Chen et al., 1997). Interestingly, PAE also enhances behavioral sensitization following acute and repeated stimulant exposure in late adolescence (Hannigan and Pilati, 1991b) as well as in adulthood (Barbier et al., 2009). Repeated stimulant exposure induces behavioral sensitization via enhanced sensitivity of underlying dopamine systems (for review see (Vanderschuren and Pierce, 2010); therefore PAE may result in sensitivity of dopamine systems,

but without altering the number of dopamine neurons or receptors. However, typical stimulant sensitization paradigms used to assess vulnerability to SUDs generally utilize a more extended exposure period (Robinson and Berridge, 2008) than that used in the Barbier et al (2009) study. Therefore it remains to be determined how PAE alters dopamine systems under extended drug exposure conditions.

In addition to its effects on dopamine systems, PAE has marked adverse effects on the hypothalamic-pituitary-adrenal axis (HPA) or stress axis. Both human and animal studies have demonstrated HPA dysregulation over the lifespan following PAE. PAE reprograms the fetal HPA axis such that HPA tone is increased throughout life, manifested as increased HPA activation and/or delayed, or deficient, recovery following stress, as well as altered stress signaling within the central nervous system (Haley et al., 2006, Uban et al., submitted-a, Weinberg et al., 2008). Importantly, there is significant overlap between central neurocircuitries underlying stress and dopamine systems, and significant bidirectional interactions between these systems (Cabib and Puglisi-Allegra, 2012). For example, acute stress sensitizes healthy individuals to the reinforcing effects of substances, and can induce relapse after prolonged abstinence (Sarnyai et al., 2001). Conversely, acute drug exposure activates stress systems, and enhances sensitivity to subsequent stressors. These findings suggest that alterations in the cross-talk between dopamine and stress systems may provide a pathway for increased neurobiological vulnerability to SUDs and relapse (Koob and Kreek, 2007, Koob, 2008, Lovallo, 2006). Cross-sensitization (response is increasingly amplified) between AMPH and stress has been demonstrated in control male rats, with AMPH sensitizing not only dopamine, but also stress systems, in the brain (Barr et al., 2002). However, very little is known about the effects of PAE on the cross-talk between dopamine and stress systems, or the effects of PAE on cross-

sensitization between AMPH and stress in the female. Of note, I and others have shown that PAE differentially alters HPA responsiveness in males and females, depending on the hormonal endpoint, time course and nature of the stressor examined (Haley et al., 2006, Hannigan et al., 1990, Uban et al., submitted-a, Weinberg et al., 2008). Moreover, as noted above, our recent studies suggest that the reduction in basal DA-R expression following chronic stress observed in Controls is attenuated in PAE males and females (Uban et al., submitted-a). That is, chronic variable stress resulted in DA-R downregulation throughout the NAc and striatum in control males and females, but this effect was significantly attenuated in PAE subjects, suggesting altered cross-talk between stress and dopamine systems following PAE. However, further studies are needed to understand individual and interactive effects of PAE and sex on both stress and dopamine systems, and to elucidate mechanisms underlying the effects observed. Sex differences are important, as sex differences have been observed in both SUDs and sensitization to drugs and stress. For example, females tend to escalate their consumption of substances more quickly and at lower doses, and typically exhibit heightened behavioral sensitization compared to males (Haseltine, 2000, Hu and Becker, 2003), especially when estradiol levels are elevated (Becker and Hu, 2008). In addition there are sex differences in HPA function across species, with females typically showing greater HPA activation and resistance to negative feedback by glucocorticoids compared to males (Young, 1998).

The current study investigated the effects of repeated AMPH exposure on behavioral sensitization, as well as cross-sensitization between AMPH and acute stress in both male and female offspring from PAE and control groups. Our overarching hypothesis was that neurobiological mechanisms mediating the interaction between stimulant exposure and stress will be altered by PAE in a direction supporting a neurobiological phenotype of increased

vulnerability to SUDs. Specifically, I hypothesized that PAE will 1) augment behavioral sensitization to AMPH; 2) augment cross-sensitization with stress; and 3) alter the effects of AMPH on DA-R expression. Moreover, given the sexually-dimorphic effects of PAE on both stress and dopamine systems, I hypothesized that PAE will have sexually-dimorphic effects on both sensitization to AMPH, cross sensitization of AMPH and stress, and changes in DA-R expression.

3.2 Material and methods

Breeding of subjects

Sprague-Dawley rats were obtained from Charles River Laboratories (St Constant, PQ, Canada). Following routine quarantine (5 weeks), rats acclimated for 1 week and then handled for an additional week. Rats were pair-housed by sex in clear polycarbonate cages corn cob bedding, and covered with ventilated filter tops. Colony rooms were maintained on 12:12 hour light/dark cycle (lights on 0800 hr) at a constant temperature (20-23^{o}C). Animals were given *ad libitum* access to water and a high protein rat diet (19% Protein Extruded Rodent Diet, #2019, Teklad Global). Virgin female (225-335 grams (g); n=38) and male (275-375g; n=18) Sprague-Dawley rats were paired at 1800. Vaginal lavage at 0830 the following day was done to assess stage of estrous cycle and detect the presence of sperm. The presence of sperm indicated pregnancy and was designated as gestation day 1 (GD1). All animal use and care procedures were in accordance with the National Institutes of Health guidelines, and were approved by the University of British Columbia Animal Care Committee.

Prenatal diets and feeding

On GD1, females were single housed in a new colony room and randomly assigned to one of three treatment groups: 1) Alcohol-treated (PAE) - liquid alcohol diet, *ad libitum* (n = 13 dams); 2) Pair-fed (PF) - liquid control diet, with maltose dextrin isocalorically substituted for alcohol, in the amount consumed by a PAE partner (g/kg/body wt/day of gestation), which controls for the reduced food intake typical in alcohol-consuming dams (n = 12 dams), 3) control (C), pelleted form of control diet consumed *ad libitum* (n = 13 dams). All dams had *ad libitum* access to water. Alcohol containing liquid diets were formulated in our laboratory to provide optimal nutrition, with 36% of the total calories derived from ethanol (Dyets Inc. Bethlehem, PA, USA), using commercial 95% ethyl grain ethanol (Commercial Alcohol Inc., ON, Canada, catalog no. P210EAAN). Fresh diets were presented and weighed daily (1800-1900 hr). This feeding schedule maintains the normal corticoid circadian rhythms in the Pair-fed dams, as providing a restricted ration effectively sets up a "meal-feeding" schedule, which re-entrains the corticoid rhythm to the feeding time rather than the light cycle (Gallo and Weinberg, 1981, Krieger, 1974). Experimental diets were provided to dams until GD 21, at which time they were replaced with laboratory chow *ad libitum* (19% Protein Extruded Rodent Diet, #2019, Teklad Global). All pregnant females were handled on GDs 1, 7, 14 and 21 for routine cage changing and weighing. In the present study, pregnant females consumed an average of 0.13, 0.14, and 0.14 (g/kg body wt/day) for gestation weeks 1, 2 and 3, respectively. Previous studies that have employed the same breeding and feeding protocols found mean blood alcohol levels in dams of ~144.4 ± 31.3 mg/dl during the third week of gestation (Hellemans et al., 2010b, Uban et al., 2010).

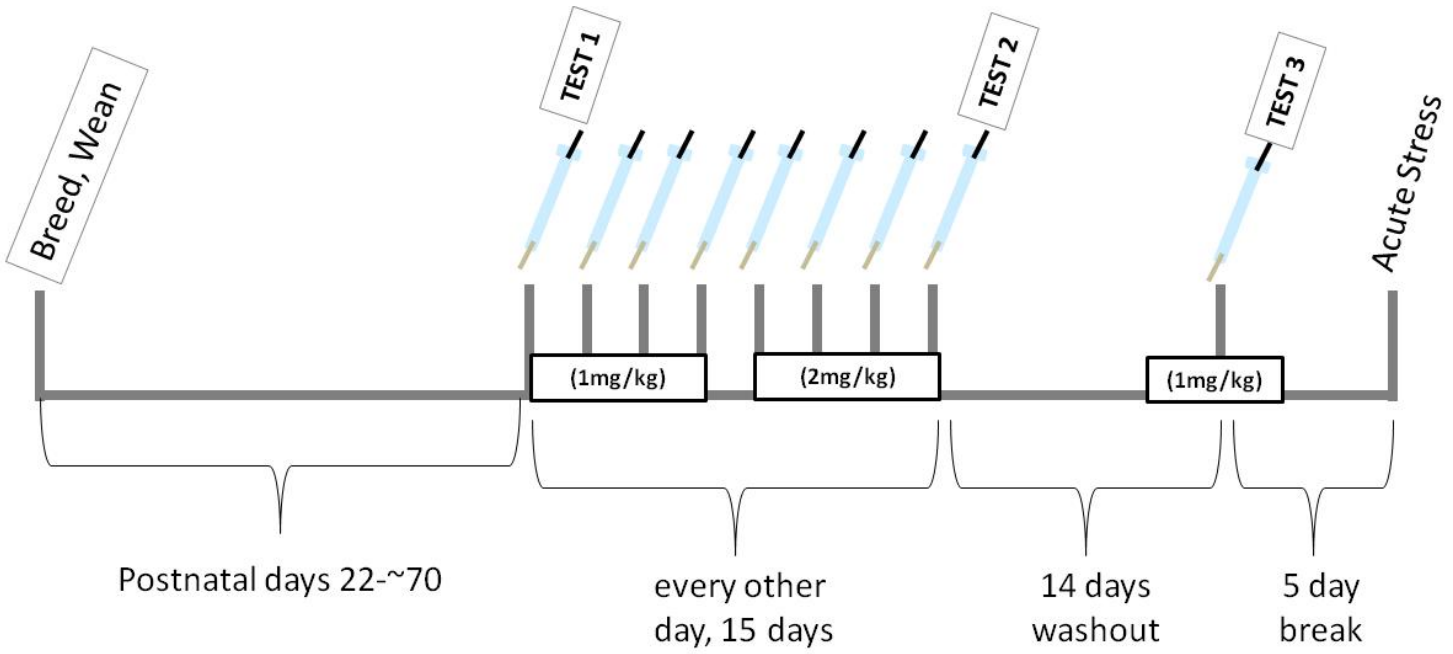
On postnatal day 1 (PND 1), pups were weighed and all litters were culled to 10 (5 females and 5 males when possible) to control for any confounding effects of litter size or sex

ratio. Dams and pups were weighed on PND 1, 8, 15 and 22. Pups were weaned on PND 22 and group-housed by litter and sex, with *ad libitum* access to the high protein rat diet and water for the remainder of the experiment. On PND 35, offspring were pair-housed by sex and prenatal group, but with a non-litter mate, and provided with a square enrichment tube for the duration of the experiment.

Amphetamine sensitization paradigm

In adulthood (70±2.5 days), each set of pair-housed rats was randomly assigned to one of two drug conditions (AMPH- or saline-treated). All subjects received injections (i.p.) every other day for 16 days (Figure 3.1). This paradigm of sensitization was adapted from (Barr et al., 2002); and given the enhanced sensitivity of our subjects following prenatal manipulations (PAE and pair-feeding) as well as the inclusion of females subjects that are known to be more sensitive to AMPH (Robinson et al., 1982), I halved the AMPH doses throughout. Thus, AMPH (*d*-Amphetamine hemisulphate salt; Sigma Aldrich, England, UK) -treated rats received increasing doses of drug: 1mg/kg for the first 4 injections, and 2 mg/kg for the last 4 injections. These low doses of AMPH were chosen to prevent ceiling effects in behavioral responsivity in PAE subjects that were expected to show increased sensitivity to stimulant exposure (Barbier et al., 2009, Hannigan, 1996). Additionally, repeated administration, compared to a single exposure, of AMPH has been shown to produce robust behavioral activation (Robinson, 1984). A 'washout' period allows for investigation of long-lasting sensitivity of underlying dopamine systems (Vanderschuren and Pierce, 2010). Thus, following the eighth injection, all subjects remained undisturbed for a 2 week washout period, at the end of which all subjects (both those previously receiving amphetamine and those previously receiving saline) were given a single injection of

Figure 3.1. Experimental Timeline



Experimental Timeline. In adulthood, offspring were exposed to 8 injections, every other day. Rats were randomly assigned to: 1) AMPH exposure (increasing doses of AMPH from 1mg/kg to 2mg/kg) or 2) saline exposure. Following injections, subjects were given a 2 week washout period, and were then administered AMPH (1mg/kg) regardless of previous drug condition. Five days later, subjects were sacrificed under either: 1) basal or 2) acute stress conditions.

AMPH (1 mg/kg, i.p.) All injections occurred at 0900-1330 hr, in order to capture the nadir of the corticosterone (CORT) circadian rhythm. Subjects were moved to a separate procedure room with contextual cues different from those of the home cage and colony room (i.e. dimmed lighting, novel (Carefresh) bedding, single housing for 80 min), as development of DA sensitivity is strengthened by contextual cues, and provides increased face validity to the human experience (Pierce and Kalivas, 1997, Wang et al., 2010). Filming of open field behavior for assessment of behavioral activation (see below) also occurred in the procedure room with novel contextual cues.

Behavioral measures

Behavior was filmed on the first and eighth injection days (days 1 and 15 of testing), as well as on the final test day following the two week washout period (day 29 of testing) when all subjects received the 1 mg/kg dose of AMPH. Behavior was tested in an open field (41 cm^3) with black plastic boarding covering all sides and the top left open to allow for overhead video recording. Sessions consisted of a baseline assessment (20 min), followed by a post-injection assessment (60 min) to examine a range of behaviors related to dopaminergic function. Rats were weighed six times throughout the testing period beginning the day before the first injection (day 0), and then on days 4, 8, 12, 14 and 28, in order to adjust the AMPH dose for body weight.

Each session was recorded from an aerial view using a digital video camera (SONY Handycam DCR-SR68). Four different aspects of behavior were assessed: 1) total distance travelled; 2) frequency of rears; 3) total number of rotations (360°); and 4) degree of stereotypy. 'Distance' and 'rotations' were quantified by ANY-maze video tracking software (version 4.75) in 5 minute blocks. 'Rearing' and 'Stereotypy' were quantified manually in 10 minute blocks,

and all experimenters were blind to experimental conditions. The co-rater reliability was > 90%. The level of 'stereotypy' exhibited was scored with a 10 point likert scale modified from (MacLennan and Maier, 1983). The behaviors scored ranged from typical (score of 1-3 to highly atypical (score of 7-10) behaviors, with midrange scores (score of 5-6) indicating the transition from typical to atypical behaviors, i.e., typical behaviors that are enhanced or exaggerated. It is important to note that *typical* behaviors include locomotion and rearing, with augmentations in these behaviors marking the transition from typical to atypical, whereas varying degrees of atypical behaviors, such as repeated rotations and restricted (stereotypic) head movements, mark the transition from moderate to severe *atypical* behaviors (MacLennan and Maier, 1983). The sensitization paradigm utilized in this study allows for examination of: 1) endogenous sensitivity of dopamine systems, which would be exhibited as an augmented response on day 1 in AMPH treated rats; 2) level of behavioral activation on day 15 in AMPH-pretreated rats; and 3) enhanced behavioral activation on day 29 in AMPH-pre-treated rats. On the final behavioral test day (day 29), 70 min post-AMPH injection, blood samples were also collected from the tail vein for analysis of plasma CORT levels.

Subsequent stress test

Following behavioral testing on day 29, subjects were given a 4 day rest period to allow for subsequent testing of stress reactivity without significantly confounding the results by previous AMPH exposure. The next morning (day 34), subjects were decapitated (0900-1030) either immediately (within 30 sec) upon removal from the colony room (basal sample) or following 30 min restraint stress in an adjacent room, and trunk blood, brains, adrenals and spleens were collected (modified from (Barr et al., 2002).

Blood collection

Blood samples (from the final behavioral test/injection day (d 29), and the acute stress day (d 34)) were collected into tubes containing 100 µl EDTA to prevent coagulation, and centrifuged at 3200 rpm for 15 min at 4 °C. Plasma was immediately transferred into 1.5 ml Eppendorf tubes and stored at -80 °C until assayed.

Brain collection and preparation

Following decapitation, whole brains were immediately extracted under RNAse free conditions, flash frozen over dry ice, wrapped in parafilm, then covered in aluminum foil, and stored at -80°C. Brains were sectioned on a cryostat (MICROM HM 505 E), to obtain coronal sections (30 µm, Bregma: 4.00 -7.32mm), with every fifth section mounted onto chilled glass slides, warmed for tissue to adhere to slides (5 min). Slides were stored at -80°C until processing.

Spleen and adrenal weights

Following brain removal, spleens and adrenal glands were promptly removed and weighed individually on a Mettler analytical balance.

Lavage samples

Smooth glass eye-droppers containing physiological saline were used for vaginal lavage immediately following behavioral testing, as well as *post-mortem* prior to organ collection. Lavage samples were placed onto glass slides, allowed to dry overnight, and then lightly stained with Toulidine Blue (1%). Cytology was determined under a light microscope (40x) as described

in (Uban et al., 2012) to determine the stage of the estrous cycle (e.g: proestrus, estrus, diestrus I or diestrus II).

Radioimmunoassays (RIA)

Corticosterone. Total CORT levels (bound plus free) were determined using an ImmuChemTM Corticosterone I125 RIA Kit (MP Biomedicals, Cat. # 07-120103). The specificity of the antibody to CORT is 100%, with a minimum detectable CORT concentration of 7.7 ng/mL. The intra- and interassay coefficients of variations were 7.1% and 7.2%, respectively.

Testosterone (T). ImmuChemTM Testosterone I125 RIA Kit (MP Biomedicals, Cat. # 07-189102) was used to measure concentrations of the total unconjugated form of T in males only. The antibody is 100% specific for T and has less than 0.01% cross-reactivity with estradiol-17β, CORT and progesterone. The minimum detectable T concentration was 0.1 ng/mL. The intra- and interassay coefficient of variations were 6.0% and 7.5%, respectively.

Estradiol (E_2). Siemens/DPC Estradiol Double Antibody RIA kit was used to determine E_2 levels in females only. The antiserum cross-reacts 100% for E_2 without detectable cross-reactivity with CORT or aldosterone. The minimum detectable concentration was 8 pg/mL. The intra- and interassay coefficient of variations were 7.0 and 8.1%, respectively.

Progesterone (P_4). ImmuChemTM Progesterone I125 RIA Kit (MP Biomedicals, Cat. # 07-107105) was used to determine P_4 levels in females only. The antibody is 100% specific to P_4 with no detectable cross-reaction with CORT or aldosterone. The minimum detectable P_4

concentration was 0.10 ng/mL. The intra- and interassay coefficient of variations were 6.4 and 2.4%, respectively.

Immunohistochemistry. Fluorescent double-staining for dopamine receptors (D_1 and D_2) was performed on half of the subjects tested for behavior (i.e. subjects subjected to restraint stress; n=7-10 per experimental group for IHC). For each subject, every 5^{th} section from the mPFC (3.72 – 2.52mm), NAc and striatum (2.52 – 1.20mm) was analyzed (Paxinos and Watson, 2005a). Glass slides containing mounted tissue were removed from -80°C freezer and laid flat at room temperature 15 minutes prior to processing. A circle was traced around the tissue on the slide using a Super HT Hydrophonic Pen (Research Products International Corp.). Sections were post-fixed for 30 min. in 3.7% paraformaldehyde in phosphate buffer solution (PBS; pH = 7.4). Sections were rinsed (3 x 10 min.) in TBS (0.1 M tris-phosphate buffer in 0.9% saline; pH 7.4), and then blocked in 4% goat serum (NDS) in TBS + 0.3% Triton-X, (Vector Laboratories, Burlington, Ontario, Canada) for 2 hr. Slides were then tapped to remove liquid, and incubated in mouse monoclonal Anti-Dopamine D_1 Receptor (1:450, Novas Biologicals) and rabbit polyclonal Anti-Dopamine D_2 Receptor (1:300, Millipore Canada) in a Nunc box lined with moistened Benchkote© with TBS at 4ºC for 22 hr on a shaker. Slides were rinsed in TBS (3 x 10 min.) followed by incubation in goat anti-mouse Alexa 594 for D_1 with goat anti-rabbit Alexa 488 for D_2 for 1 hr (1:450; Invitrogen, Burlington, Ontario Canada). Sections were then rinsed in TBS (3 x 15 minutes), followed by a brief dip in dH_20, and were left to dry for 3 hours prior to being cover-slipped with 2.5% PVA-DABCO (Sigma, Oakville, Ontario, Canada) and stored in the dark at 4^oC until imaging. These D_1 and D_2 antibodies have been previously assessed for specificity (Oda et al., 2010, Rajput et al., 2009). In the present study, controls included a series

of slides involving omission of the primary and/or secondary antibodies to assess for specificity of binding using optical density measurements (Table 2.1).

Quantification of data

Densometric Analysis. The experimenter was blinded to treatment conditions while performing analysis. Images of D_1 and D_2 receptor expression were acquired with an Olympus FV1000 confocal microscope (20x) and analyzed with ImageJ (Rasband, 1997-2011). In order to avoid saturation, imaging parameters were set using the 5 brightest immunofluorescence subjects within a region. Imaging only the middle 1.14 μm of each section, the following settings were found to produce optimal images: D_1 corresponded with the laser wavelength 568 (mPFC: laser intensity=20%, HV=760, offset=8%; NAc and Striatum: laser intensity= 20%, HV=790, offset=8%). D_2 corresponded with the laser wavelength 488 (mPFC: laser intensity=3.5%, HV=745, offset=8%; NAc and Striatum: laser intensity= 3.5%, HV=745, offset=8%). Background measurements were obtained from the glass slide adjacent to the brain section to control for differential background on slides, as D_1 and D_2 are widely distributed throughout the brain. Corrected optical density values were averaged across right and left hemispheres and across sections (i.e. 6 measures per subject per subregion).

Statistical Analyses

All statistical analyses were performed using Statistica 10.0 software (StatSoft, Inc), with analyses of variance (ANOVAs) followed by Newman-Keuls *post-hoc* analyses. Developmental data were analyzed using repeated-measures ANOVAs (RM-ANOVA) with prenatal group (Control, Pair-fed, PAE) as the between-subjects factor, and GD or lactation day (LD) as the

within-subjects factor for the dams, or postnatal day (PND) as the within-subjects factor for the offspring. A separate ANOVA was run to assess pup body weights at birth (i.e. GD 1). Male and female offspring were analyzed together for developmental data, but separately for all other analyses. Adult body weight was measured throughout the experiment on days 0 (i.e day before first injection), 4, 8, 12, 14 and 28, and percent change from day 0 was analyzed as a RM-ANOVA, with prenatal group and drug condition as between group factors, and day as the within-subject factor. For behavioral data, RM-ANOVAs were used to analyze each behavioral measure, with prenatal group and drug condition as between subject factors, and day (1, 15, 29) and time block as within-subject factors. Serum samples from day 29 following AMPH exposure were analyzed for CORT levels by ANOVA, with prenatal group and drug condition as between subjects factors. Serum samples at termination (day 34) were analyzed for ACTH, CORT, T, P_4 and E_2 levels. Hormone levels were analyzed by RM-ANOVAs, with prenatal group, drug condition, and stress condition as between subject factors, and hormone as a within-subject factor. DA-R optical densities were analysed by RM-ANOVA, with prenatal group and drug condition as between-subject factors and subregion (i.e. mPFC: PL, IL; NAc: core, shell) and DA-R subtype (D_1, D_2) as within-subject factors. For all data analyses on adult females, stage of estrus was used as a covariate, as estrous stage is known to interact with endocrine and dopamine systems (Becker et al., 2001). If stage of estrus was not a significant covariate, the data were re-analyzed without controlling for estrous stage.

Tests of *a priori* hypotheses utilized a Bonferroni correction. *A priori,* I hypothesized that AMPH treatment will: 1) enhance behavioral activation compared to saline exposure; 2) enhance subsequent stress responsivity (Barr et al., 2002); and 3) be augmented in PAE compared to control subjects.

3.3 Results

Developmental Data

Reduced body weights in PAE and PF compared to C dams during gestation and lactation: Analysis of maternal weight gain throughout pregnancy revealed a significant main effect of day ($F_{7, 217}$= 256.24, p<0.001), a trend for an effect of prenatal group ($F_{2,217}$=3.01, p=0.06), and a significant interaction between prenatal group and day ($F_{14, 217}$= 5.03, p<0.001) (Table 3.1). *Post-hoc* analyses revealed, as expected, lower body weights in PAE and PF compared to C dams throughout gestation, and on LDs 1, 7, and 14 (p's<0.045), and lower body weights in PAE compared to PF and C dams on GD 21 (p<0.01).

No differences in pup body weights among prenatal groups: At birth, there was a main effect of sex ($F_{1, 31}$=551.17, p<0.001), with males weighing more than females, but no significant differences among prenatal groups in pup birth weights ($F_{2,31}$=1.54, p=0.22) (Table 3.1). Throughout the pre-weaning period, there were significant effects of PND ($F_{3, 93}$=141.53, p<0.001) and sex ($F_{1, 93}$=12.93, p<0.001), and a significant PND x sex interaction ($F_{3, 93}$=35.28, p<0.001), but no effect of prenatal group ($F_{2, 93}$=0.64, p=0.53). All pups gained weight during the preweaning period, and overall body weight was greater in males compared to females on PNDs 1 and 14 (p's<0.013).

Table 3.1 Developmental Data

Dam Body Weights	***Prenatal Treatment***		
	Control	**Pair-fed**	**Alcohol Exposed**
Pregnant Dams (N)	10	12	12
Dam Weight (g)			
GD1	295 ± 10	275 ± 8 [a]	282 ± 8 [a]
GD7	327 ± 11	297 ± 7 [a]	299 ± 8 [a]
GD14	375 ± 12	330 ± 13 [a]	334 ± 8 [a]
GD21	460 ± 15	425 ± 9 [b]	404 ± 8 [b]
LD1	379 ± 15	342 ± 9 [a]	329 ± 9 [a]
LD7	367 ± 12	338 ± 10 [a]	345 ± 7 [a]
LD14	367 ± 8	336 ± 15 [a]	354 ± 7 [a]
LD22	342 ± 8	329 ± 7	340 ± 6

	Males			**Females**		
Offspring Body Weights						
	Control	**Pair-fed**	**PAE**	**Control**	**Pair-fed**	**PAE**
PND1	6.8 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.5 ± 0.8	6.3 ± 2.1	5.6 ± 1.9
PND22	55.0 ± 1.4	55.8 ± 1.0	56.2 ± 1.5	54.2 ± 0.7	54.0 ± 1.2	54.7 ± 1.2

Dam and offspring body weights: [a]PF=PAE<C. [b]PAE<PF<C. Abbreviations: GD, gestation day; LD, lactation day; PND, postnatal day.

Outcome Measures in Adulthood

Attenuated weight gain in PAE and AMPH-treated rats over the 28 day experimental period: There were significant prenatal group x day ($F_{8, 908}$=3.5, p=0.05), drug condition x day ($F_{4, 908}$=4.19, p<0.002), and sex x day ($F_{4, 908}$=118.248, p<0.001) interactions (Figure 3.2). *Post-hoc* analysis revealed reduced weight gain in PAE compared to C and PF rats on days 8-28 (p's<0.007), but not on day 4 (p's>0.23). Overall, AMPH-exposure attenuated weight gain on days 12 and 14 compared to that in saline-treated rats (p's<0.001).

Display of enhanced typical behaviors (behavioral activation) in PAE subjects after repeated exposures to amphetamine

PAE males and females showed greater locomotor behavior (greater distance travelled) than C and PF rats following repeated AMPH exposure: In males, there was a significant four-way interaction of prenatal group x drug condition x day x time block ($F_{52, 2756}$=1.41, p=0.03), as well as significant three-way interactions (prenatal group x day x time block, drug condition x day x time block; all p's <0.001), and two-way interactions (drug condition x day, drug condition x time block, day x time block; all p's<0.001), and significant main effects of drug condition, day, and time block (all p's <0.001), and a trend for prenatal group (p=0.06; Figure 3.3A). *Post-hoc* analyses revealed, as expected, significantly greater distances travelled in AMPH- compared to saline-treated males across prenatal groups on both day 1 (C: 20-50 min; PF: 25 and 35-40 min; PAE: 25-50 min), and day 15 (C: 35 min; PF: 50 min; PAE: 15 and 25-50 min).

Figure 3.2. Percent change in adult body weight

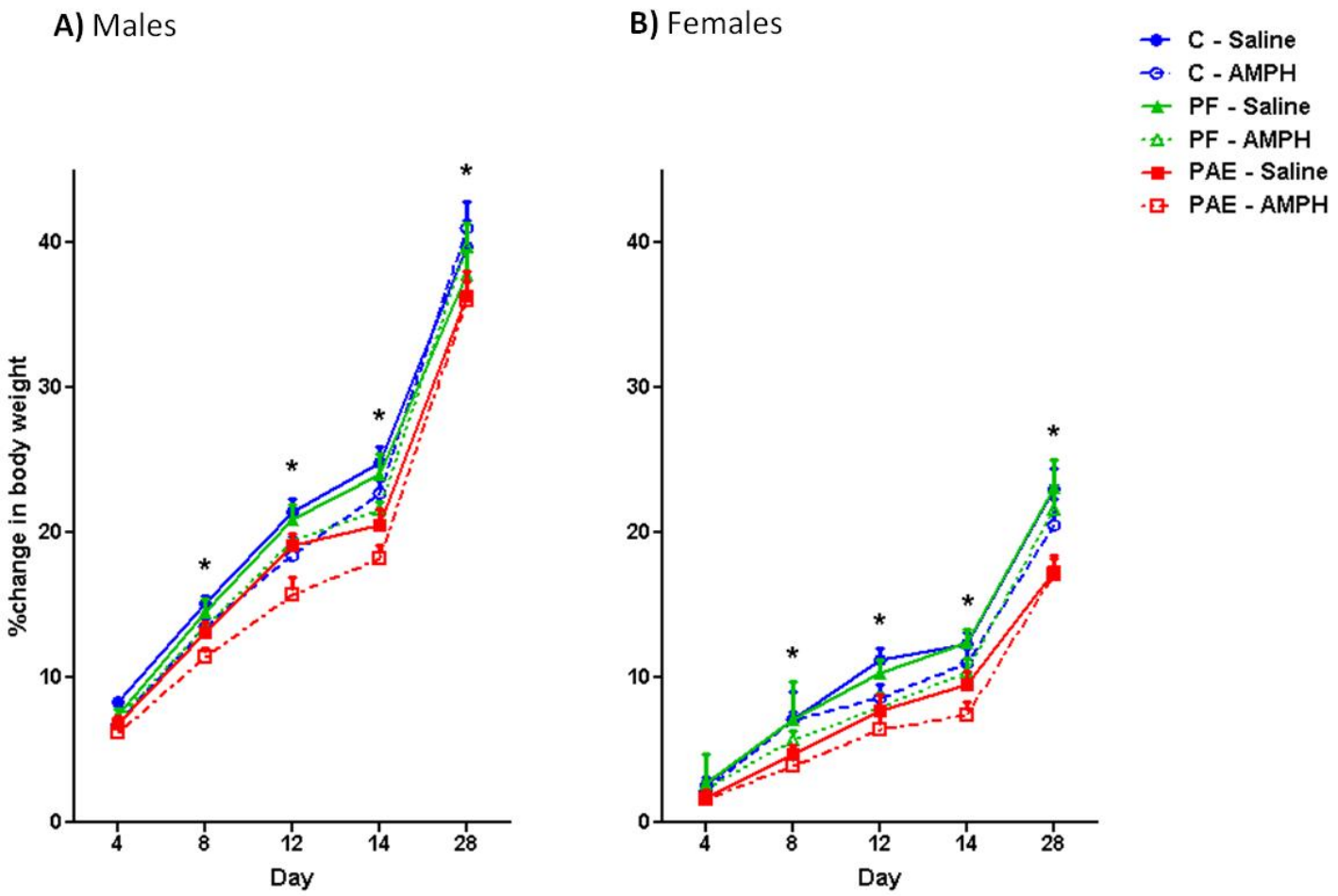
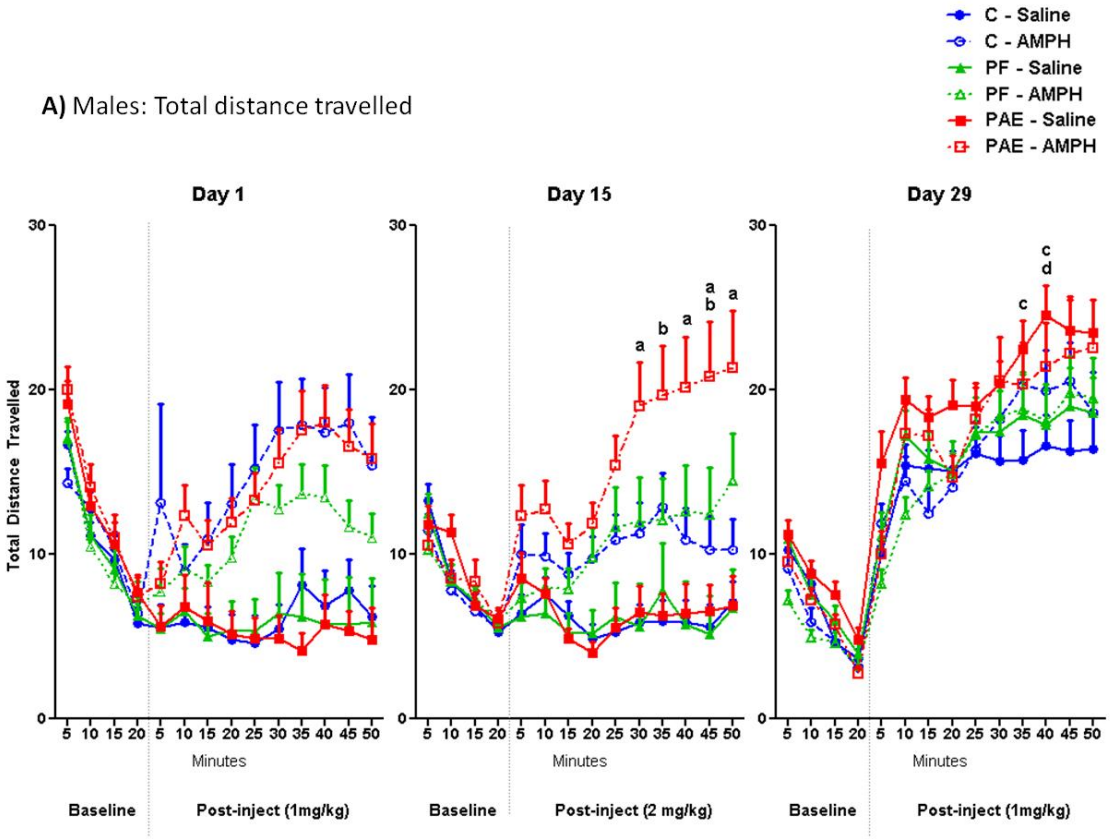


Figure 3.2. Percent change in adult body weight. Mean±SEM. Starting from the day before the first injection (day 0), percent change in body weight was calculated in : **A) Males, and B) Females** until day 28 (i.e. day before last behavioral test)**.** *AMPH<saline-exposed (main effect of drug condition) and PAE<C (main effect of prenatal group).

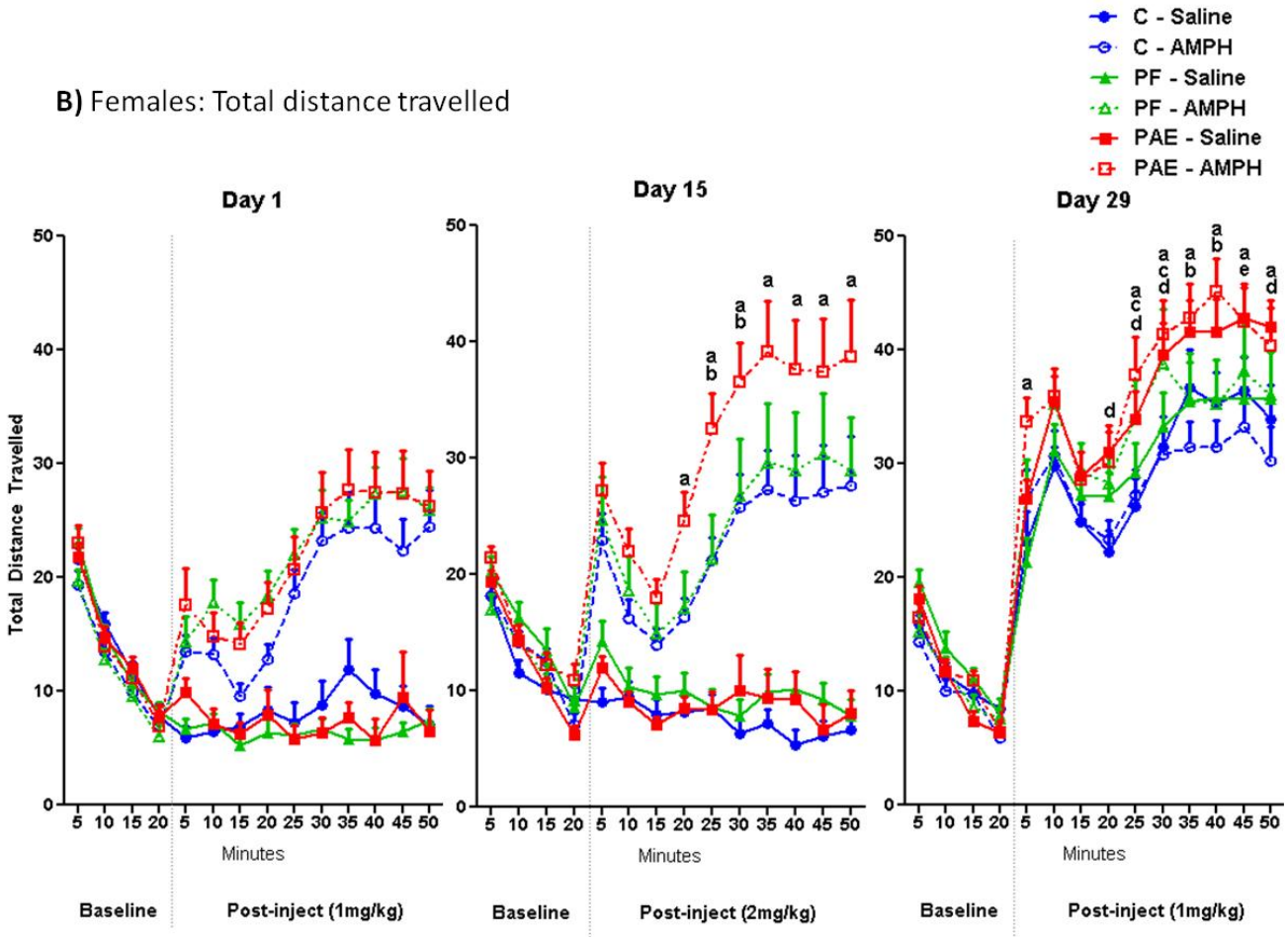
However, PAE AMPH-treated males travelled greater distances on day 15, after 8 injections of AMPH, compared to both AMPH-treated C (time blocks 30 and 40-50 min), and PF (35 and 45 min; p's<0.05) males, suggesting enhanced locomotor activity at an earlier time point in PAE compared to control males. Intriguingly, saline-pretreated PAE males that received AMPH for the first time on day 29 travelled greater distances compared to both C (35-40 min) and PF (40 min; p's<0.05) males pre-treated with saline, indicating that PAE males also demonstrate enhanced behavioral activation on their first AMPH exposure following the mild stress of repeated i.p. saline injections.

In females, there were significant three-way interactions (prenatal group x day x time block, drug condition x day x time block; all p's<0.003), and two-way interactions (drug condition x day, prenatal group x time block, drug condition x time block, day x time block; all p's<0.001), and significant main effects of prenatal group, drug condition, day, and time block (all p's<0.003; Figure 3.3B). Consistent with our hypotheses, *a priori* analyses revealed significantly greater distances travelled in AMPH- compared to saline-treated females within each prenatal group on day 1 (C: 5-10 min and 25-50 min; PF: 5-50 min; PAE: 10-50 min) and day 15 (C: 5-50 min; PF:5-50 min; PAE: 5-15 min of baseline and at 10-50 min post-injection). Importantly, on day 29, when all rats received AMPH injections, AMPH-pretreated PAE females travelled greater distances than saline-pretreated PAE females (PAE: 5 min at baseline, 5 and 15 min post-injection; p's<0.0025), and compared both to AMPH-pretreated C (day 15: 20-50 min; day 29: 5, 25-50 min) and PF (day 15: 25-30 min; day 29: 35-40 min) females on days 15 and 29 (p's<0.0025). These findings indicate that, similar to what was seen in males, PAE females showed enhanced behavioral activation at an earlier time point (day 15) than C and PF females, as well as enhanced behavioral activation on day 29. Furthermore, in saline-pretreated females

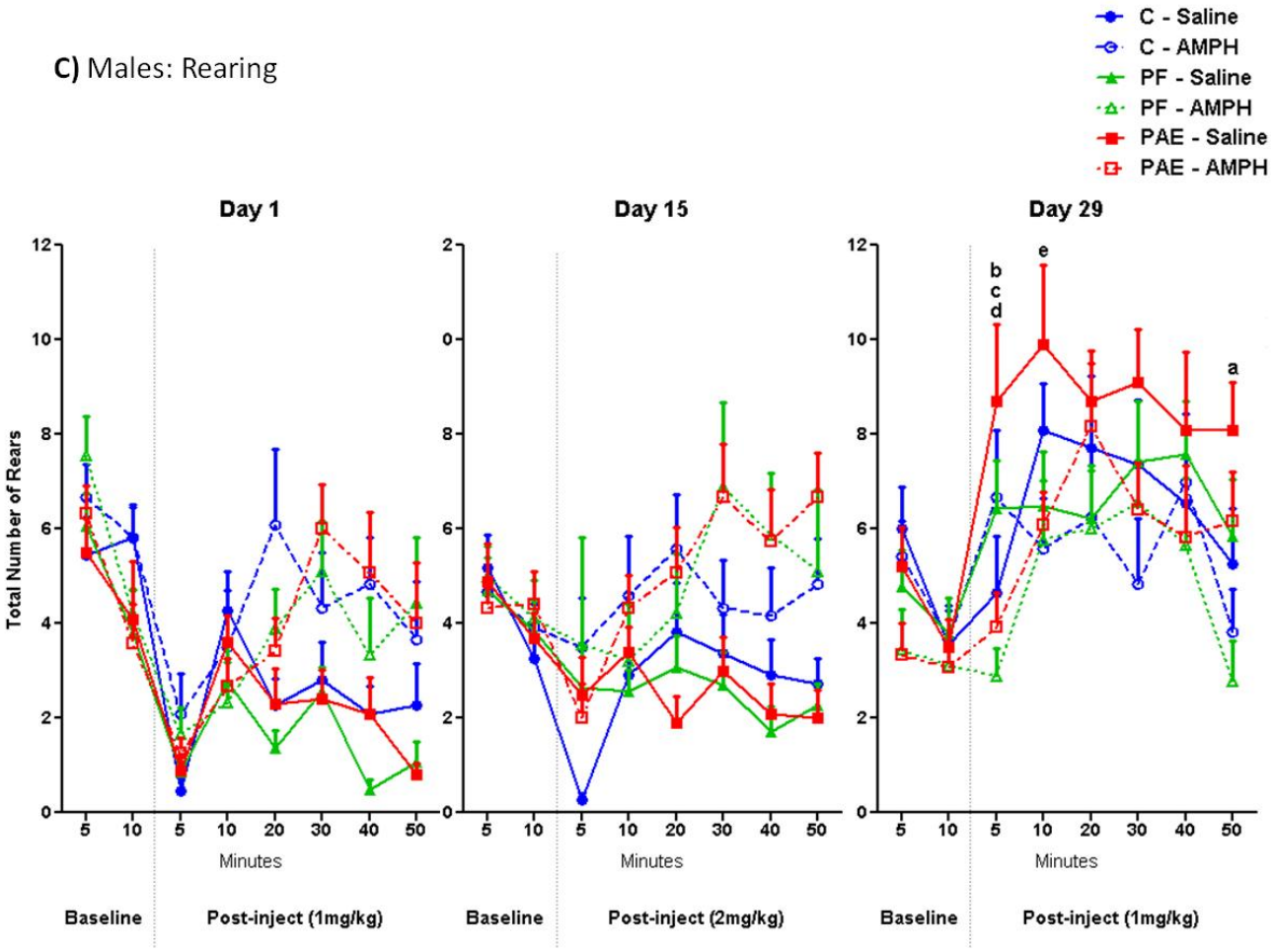
Figure 3.3. Behavioral assessment during AMPH exposure



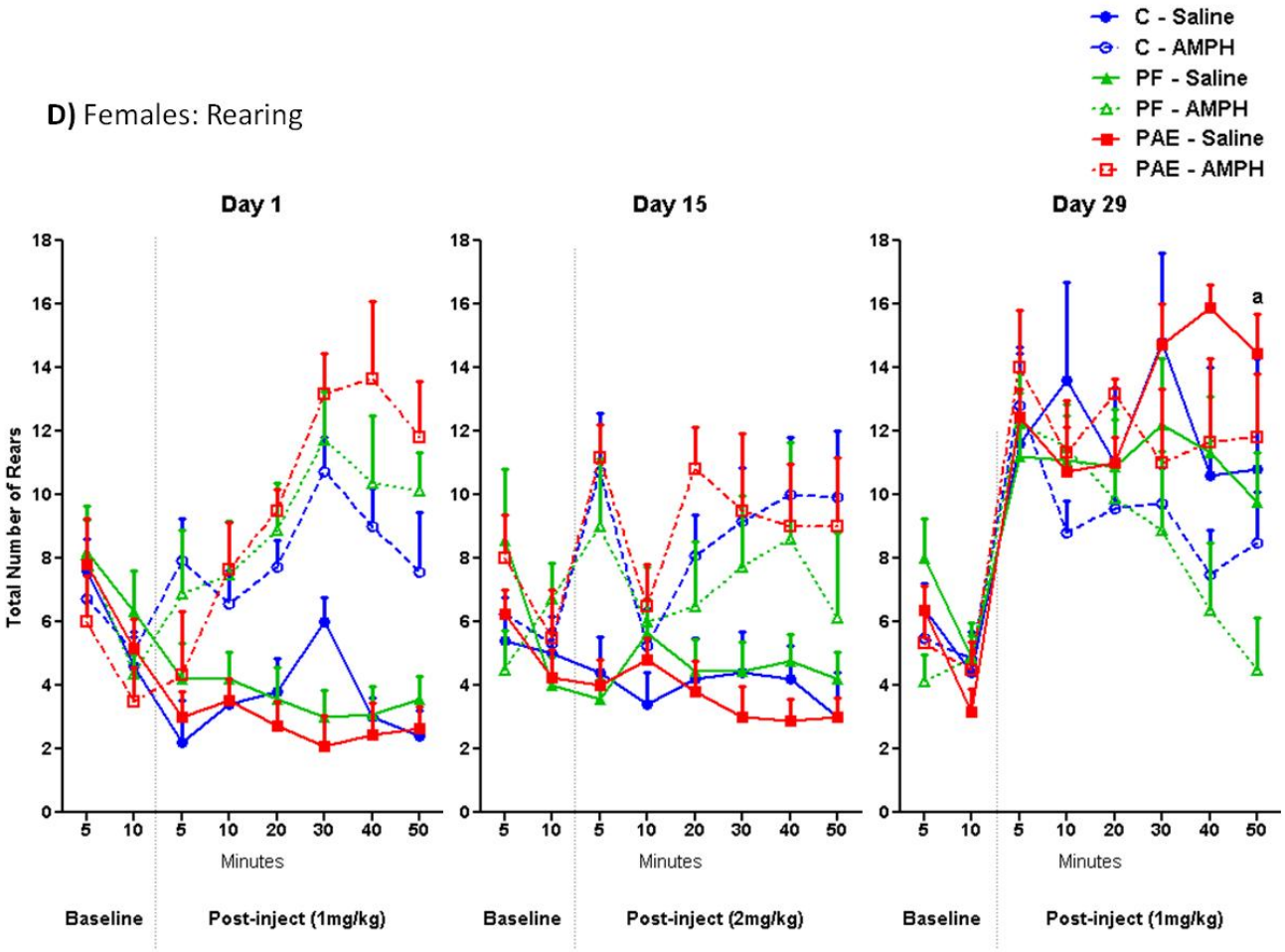
B) Females: Total distance travelled



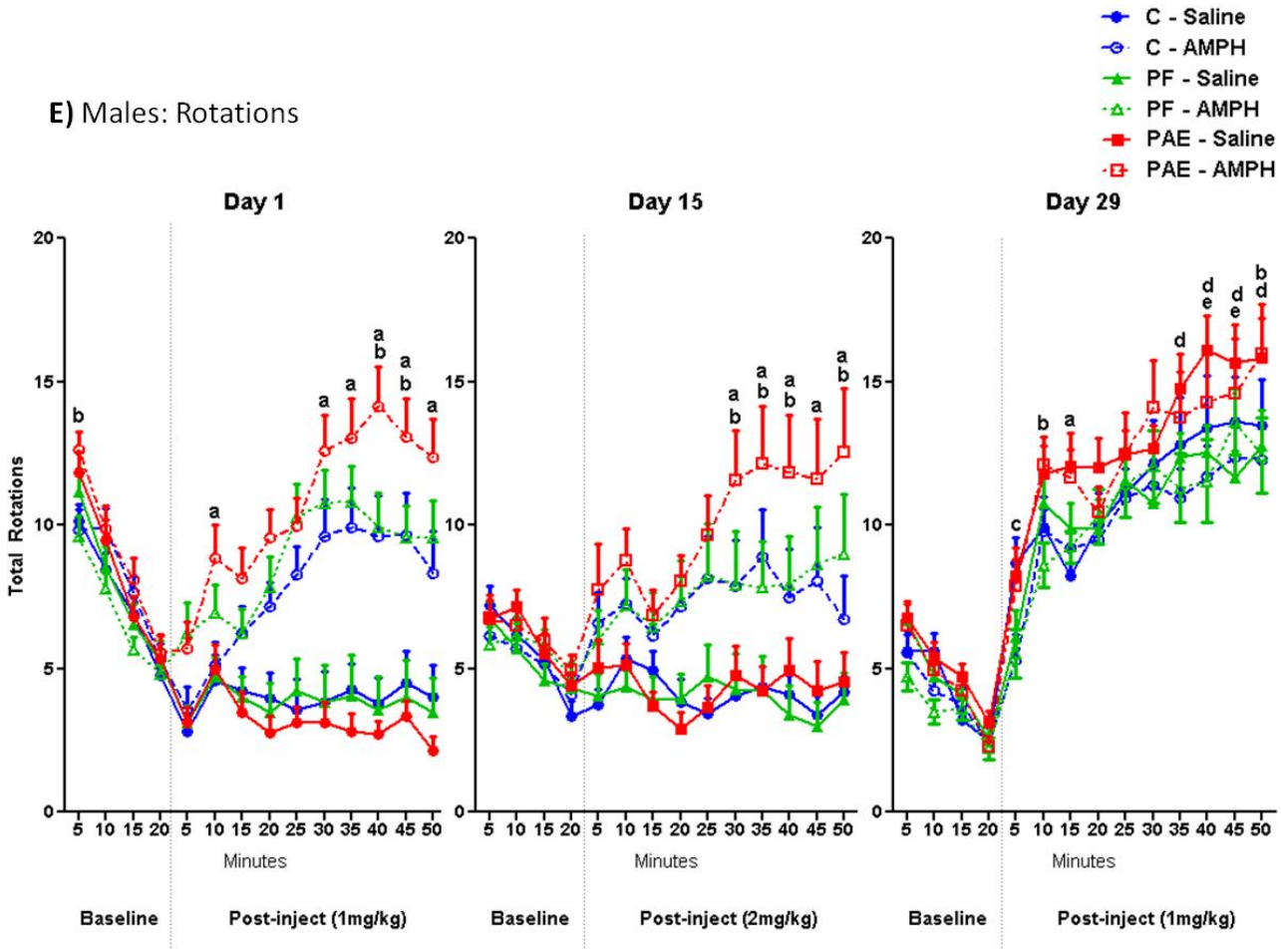
C) Males: Rearing



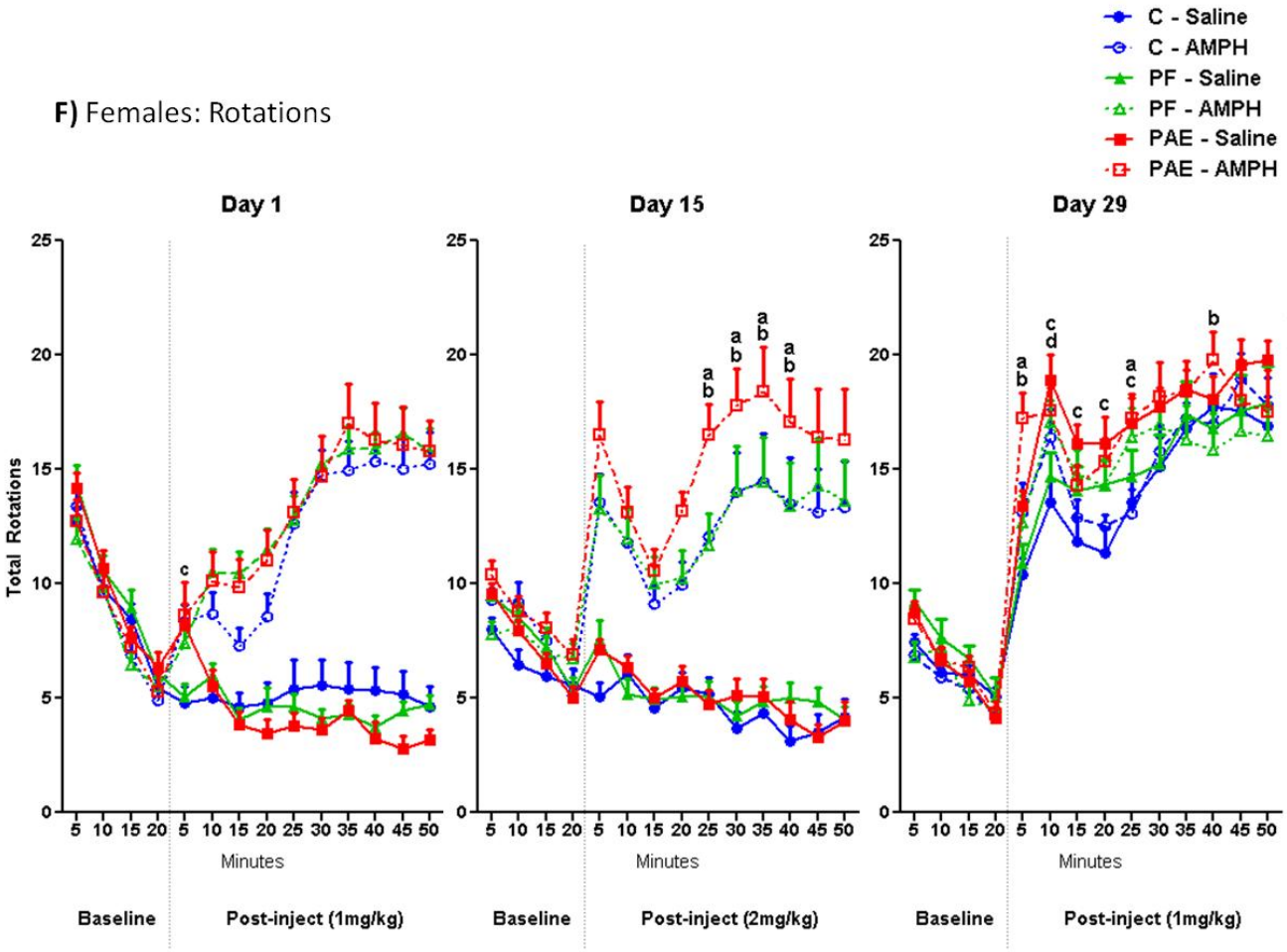
D) Females: Rearing



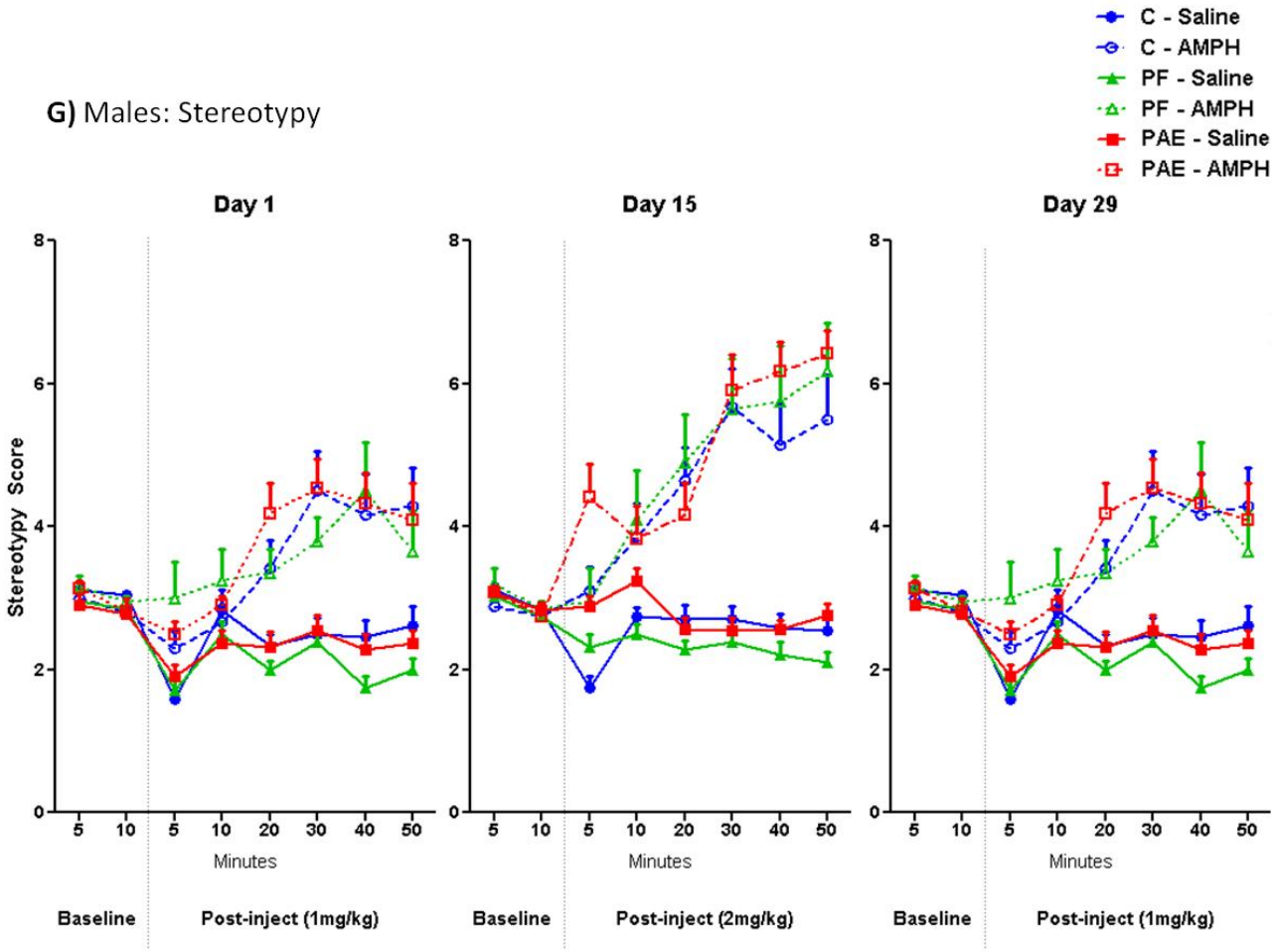
E) Males: Rotations



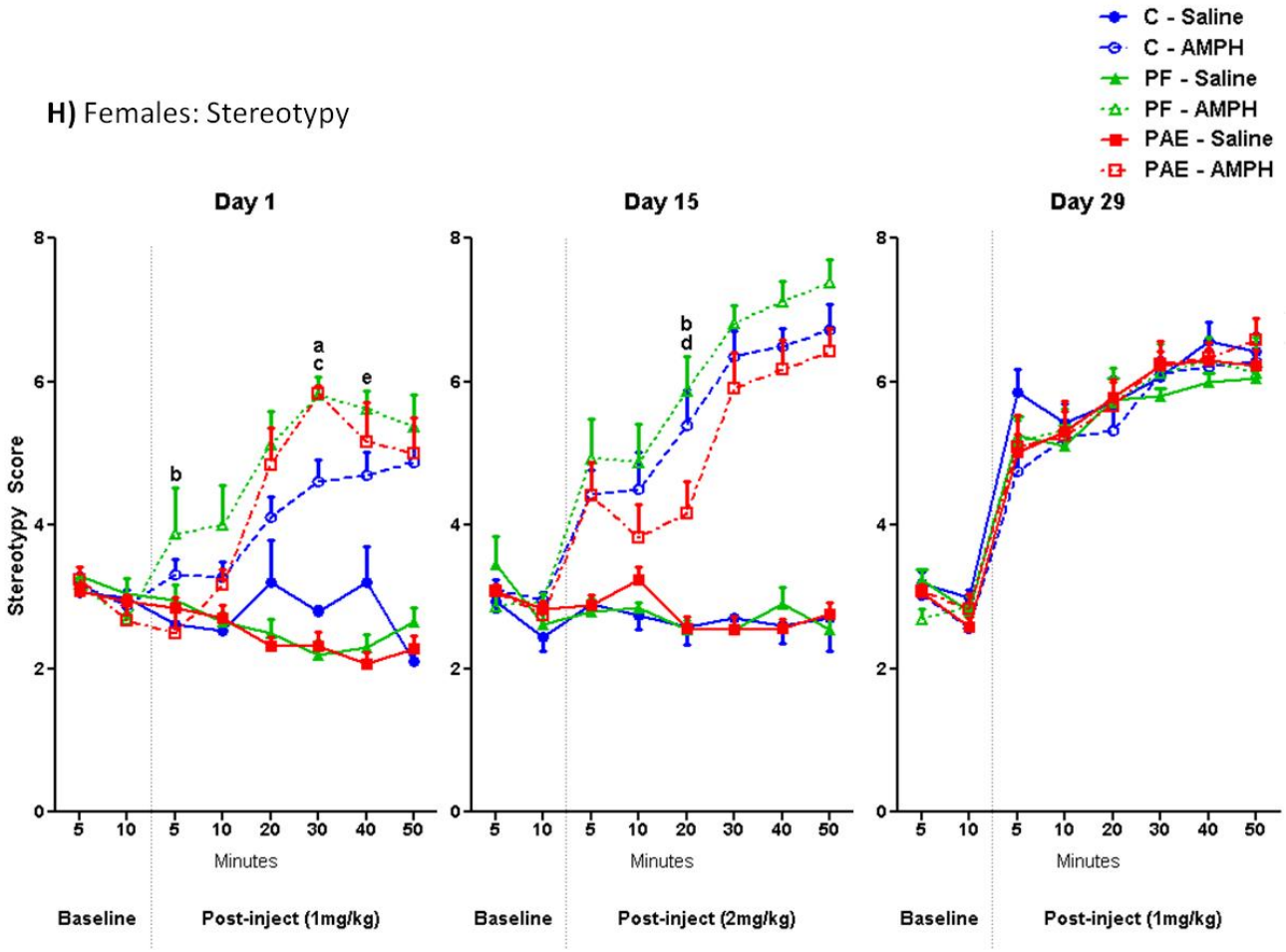
F) Females: Rotations



G) Males: Stereotypy



H) Females: Stereotypy



Behavioral assessment during AMPH sensitization paradigm. Mean±SEM. 4 different behavioral measurements tested during 80 minute session inside open field apparatus on three different test days: 1, 15 and 29. Distance travelled by **A) Males:** [a]AMPH-exposed: PAE>C; [b]AMPH-exposed: PAE>PF; [c]Saline-exposed: PAE>C; [d]Saline-exposed: PAE>PF. **B) Females:** [a]AMPH-exposed: PAE>C; [b]AMPH-exposed: PAE>PF; [c]AMPH-exposed: PF>C; [d]Saline-exposed: PAE>C; [e]Saline-exposed: PAE>PF. Total number of rears committed by **C) Males :** [a]AMPH-exposed : PAE>PF ; [b]AMPH-exposed : C>PAE ; [c]AMPH-exposed C>PF ; [d]Saline-exposed : PAE>C ; [e]Saline-exposed : PAE>PF. **D) Females :** [a]AMPH-exposed : PAE>PF. Rotations committed by **E) Males :** [a]AMPH-exposed : PAE>C; [b]AMPH-exposed : PAE>PF ; [c]AMPH-exposed : C>PF ; [d] Saline-exposed : PAE>C ; [e]Saline-exposed : PAE>PF. **F) Females :** [a]AMPH-exposed : PAE>C ; [b]AMPH : PAE>PF ; [c]Saline-exposed : PAE>C ; [d]Saline-exposed : PAE>PF. Stereotypy rating for **G) Males** and in **H) Females :** [a]AMPH-exposed : PF>C ; [b]AMPH-exposed : PF>PAE ; [c]AMPH-exposed : PAE>C ; [d]AMPH-exposed : C>PAE ; [e]Saline-exposed : C>PAE.

that received AMPH for the first time on day 29, PAE females travelled greater distances compared to both saline-pretreated C (20-30 and 50 min) and PF (45 min) females (ps<0.0025); therefore indicating enhanced behavioral activation in PAE compared to control females following to first AMPH treatment following the mild stress of repeated i.p. saline injections. Analyses further reveal that AMPH-pretreated PF females travelled greater distances than AMPH-pretreated C females on day 29 (25-30 min; p's<0.0025), suggesting that PF females also show enhanced behavioral activation to AMPH, albeit at a later time point than PAE females.

AMPH-treated rats reared more than saline-treated rats on days 1 and 15, and saline pretreated rats showed enhanced rearing to their first AMPH injection on day 29, an effect that was augmented in PAE males: In males, there was a significant three-way interaction (drug condition x day x time block ; $F_{14, 868}$=2.56, p=0.001), significant two-way interactions (drug condition x day, drug condition x time block, day x time block; all p's<0.001), and significant main effects (day, time block (all p's<0.001; Figure 3.3C). Overall, AMPH-pretreated males across prenatal groups had an increased number of rears on day 1 (40 min), and day 15 (30-50 min), whereas on day 29, saline-pretreated males reared more than AMPH-pretreated males (5 min; p's<0.003). Importantly, among saline-pretreated males who received their first injection of AMPH on day 29, PAE males reared more than both C (5 min) and PF (10 min) males (p's<0.003), providing another indicator of enhanced behavioral activation to AMPH exposure in PAE compared to control males, in this case, following the mild stress of repeated saline injections.

In females, there was a significant three-way interaction (drug condition x day x time block; p<0.001), significant two-way interactions (drug condition x day, drug condition x block, day x time block; p's<0.001), and significant main effects (drug condition, day, time block;

p's<0.002; Figure 3.3D). Overall, AMPH-pretreated females reared more than saline-pretreated females on day 1 (20-50 min) and day 15 (5 and 20-50 min) (p's<0.05), but like males, on day 29, when all subjects received AMPH, saline-pretreated females reared more than AMPH pretreated females (30-50 min; p's<0.05).

Together these findings indicate that across prenatal groups, there was enhanced behavioral activation overall in AMPH- compared to saline-pretreated males and females during the development of enhanced behavioral activation (days 1-15), but that following the two week washout period, saline pretreated subjects exposed to the mild stress of repeated saline injections showed enhanced behavioral activation to their first AMPH injection.

Display of atypical behaviors: rotations and stereotypy

PAE males and females showed more rotation behavior than their C and PF counterparts following repeated AMPH exposure: In males, there was a significant three-way interaction (drug condition x day x time block; $F_{26, 2704}$=6.43, p<0.001), significant two-way interactions (drug condition x day, drug condition x time block, test day x time block; all p's<0.001), and significant main effects (prenatal group, drug condition, day, time block; all p's<0.01; Figure 3.3E). Overall, AMPH-treated males displayed more rotations than saline-treated males on days 1 (15-50 min) and 15 (20-50 min; p's<0.05), but not on day 29 (when all rats received AMPH; p's>0.05). Furthermore, *a priori* analyses revealed that AMPH-treated PAE males rotated more than both AMPH-treated C and PF males throughout testing [i.e. day 1 (C: 10 and 30-50 min; PF: 5 min during baseline and 40-45 min post-injection); day 15 (C: 30-50 min; PF: 30-40 and 50 min), and day 29 (C: 15 min; PF: 10 and 50 min) p's< 0.0025]. In addition, on day 29, PAE

males rotated more than saline-pretreated C and PF males upon first exposure to AMPH (C: 35-50 min; PF: 40-45 min; ps<0.0025), suggesting enhanced atypical behaviors unmasked in PAE compared to C and PF males following the mild stress of repeated saline injections.

In females, there was a significant three-way interaction (drug condition x day x time block ($F_{26, 2730}$=21.17, p<0.001), significant two-way interactions (drug condition x day, drug condition x time block, day x time block (all p's<0.001), and significant main effects (drug condition, day, time block (all p's<0.001) and a trend for prenatal group (p=0.06; Figure 3.3F). As expected, there were increased rotations in AMPH- compared to saline-treated females overall on day 1 (10-50 min), day 15 (5-50 min), and day 29 (5 min) (p's<0.05). Further *a priori* analyses revealed that AMPH-pretreated PAE females rotated more than AMPH-pretreated C and PF females on day 15 (C: 25-40; PF: 25-40 min) and day 29 (C: 5 and 25 min; PF: 5 and 40 min; p's<0.0025). Furthermore, as seen in males, saline-pretreated PAE females given AMPH for the first time on day 29 rotated more than saline-pretreated C and PF females (C: 10-25 min; PF: 10 min; p's< 0.0025), once again indicating enhanced atypical behaviors in PAE compared to C and PF females to their first AMPH exposure following the mild stress of repeated saline injections.

AMPH enhanced stereotypy similarly across all prenatal groups: In males, there was a significant three-way interaction (drug condition x day x time block; $F_{14, 896}$=7.41, p<0.001), significant two-way interactions (drug condition x day, drug condition x time block, day x time block; all p's<0.001), and significant main effects (drug condition, day, time block; all p's<0.001; Figure 3.3G). There was enhanced stereotypy in AMPH- compared to saline-treated males overall on day 1 (20-50 min), day 15 (5-50 min), and day 29 (20-30 min; p's<0.05). Stereotypy ratings

were similar among prenatal groups throughout behavioral testing, with behavioral scores ranging from 4-6 following AMPH-exposure, indicative of enhanced behaviors in the typical to atypical range. Thus, the moderate doses of AMPH used in the present study did not induce high levels of atypical behaviors indicative of severe stereotypies.

In females, there were significant three-way interactions (prenatal group x drug condition x time block, drug condition x day x time block; p's<0.001), significant two-way interactions (prenatal group x drug condition, drug condition x day, drug condition x time block, day x time block; all p's<0.04; Figure 3.3H) and significant main effects (drug condition, day, and time block; all p's<0.001). *Post-hoc* analyses revealed enhanced stereotypy in AMPH- compared to saline-treated females overall on day 1 (10-50 min), and day 15 (5-50 min; p's<0.05). Additionally, AMPH-treated PF females exhibited more stereotypy than C (5 and 20-50 min) and PAE (5-20 and 50 min) females (p's<0.05). Similar to males, female behavioral scores across prenatal groups ranged from 4-6 following AMPH exposure on day 1 and 29. Interestingly, on day 15, behavioral scores of AMPH-pretreated females ranged from 6-8 during the latter half of the session, indicative of moderate levels of atypical behaviors.

A direct comparison of stereotypic behaviors in males and females revealed a significant four-way interaction (sex x drug condition x day x time block; $F_{14, 1694}$=2.09, p<0.01), significant three-way interactions (sex x prenatal group x time block, sex x drug condition x day, prenatal group x drug condition x time block, drug condition x day x time block, and sex x day x time block; p's<0.04), significant two-way interactions (day x drug condition, drug condition x time block, sex x time block, and day x time block; p's<0.001) and significant main effects (sex, drug condition, day, and time block; p's<0.001). *Post-hoc* analysis on the four-way interaction revealed enhanced stereotypy scores in females compared to males following AMPH exposure

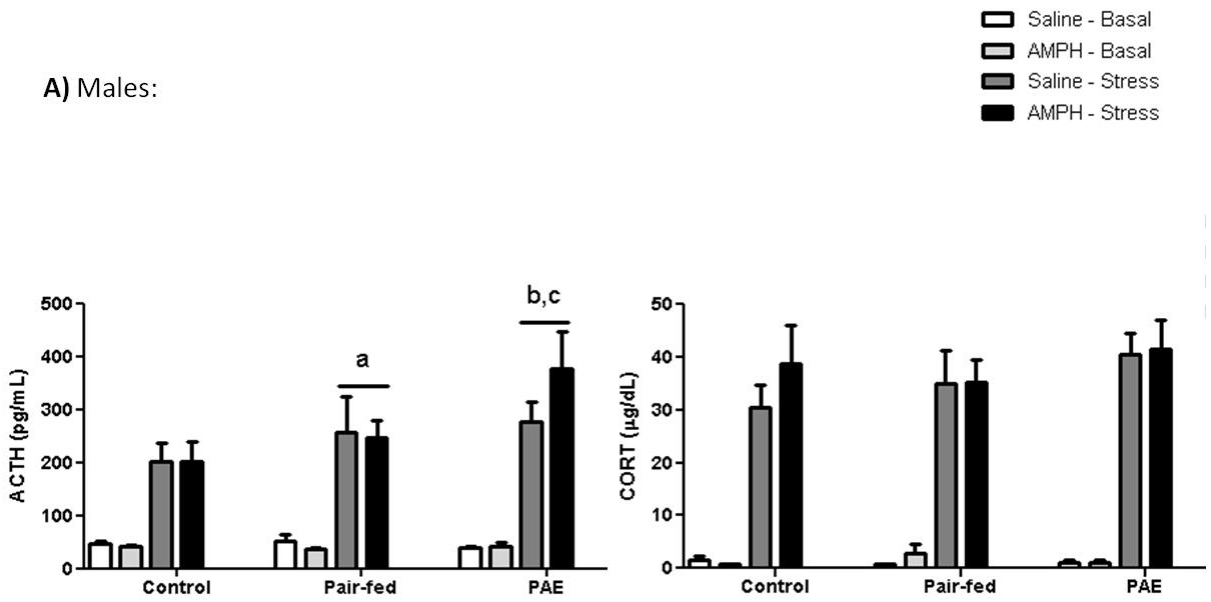
on day 1 (21-60 min), day 15 (1-60 min), and day 29 (1-10 min; p's<0.04). Enhanced stereotypy scores were also observed in saline-treated females compared to saline-treated males on day 1 (1-10 min), and on day 29 following their first exposure to AMPH after the mild stress of repeated i.p. saline injection (1-50 min; p's<0.03).

Hormones

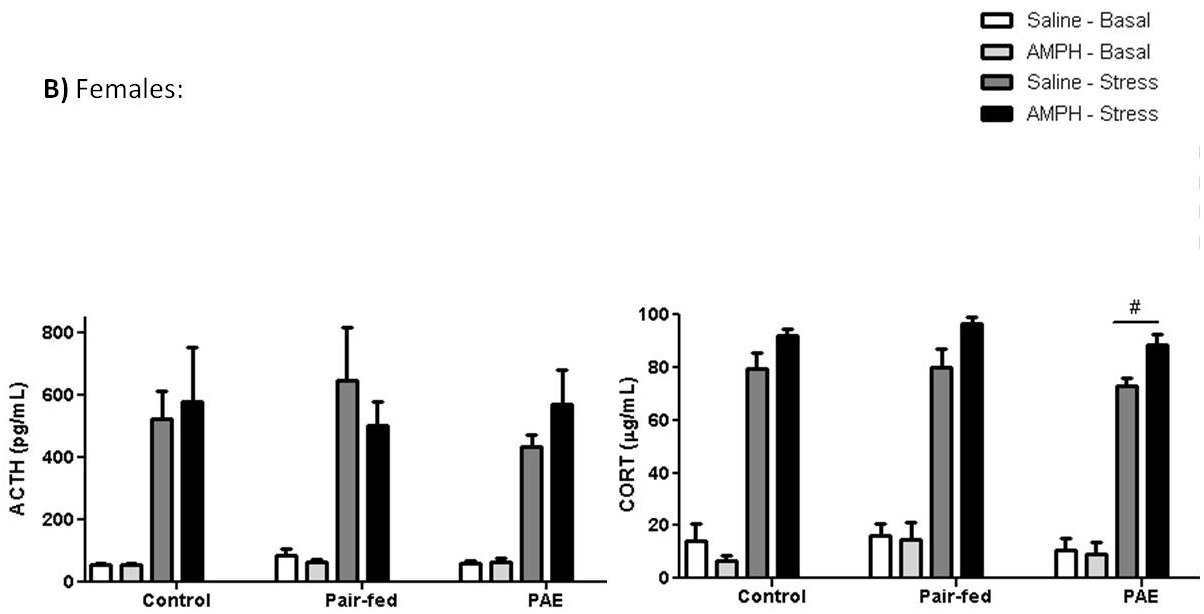
CORT levels did not differ among prenatal groups following the final AMPH injection (day 29): No significant differences among prenatal groups or between drug conditions were observed for CORT levels collected after AMPH exposure (day 29) in either males (p's>0.25) or females (p's>0.15; data not shown). In females, estrous stage was a significant covariate ($F_{1,110}$=3.96, p=0.04), where rats in proestrus/estrus (74.9± 1.7) had higher CORT levels than those in diestrus I/II (69.8±2.0).

AMPH enhanced ACTH release following acute restraint stress in PAE males and the CORT response showed a similar trend in PAE females: In males, there was a significant three-way interaction among prenatal group x stress x hormone ($F_{2,108}$=3.23, p=0.04), significant two-way interactions between prenatal group x stress ($F_{2, 108}$=3.23, p=0.04), stress x hormone ($F_{1, 108}$=87.44, p<0.001), and significant main effects of prenatal group ($F_{2, 108}$=2.94, p=0.05), stress condition ($F_{1, 108}$=134.45, p<0.001), and hormone type ($F_{1, 108}$=184.57, p<0.001; Figure 3.4A). *Post-hoc* analyses revealed enhanced ACTH levels following acute stress in PAE compared to PF and C males (p's<0.003), and in PF compared to C males, regardless of drug condition

Figure 3.4. Stress hormone levels



B) Females:



Stress hormone levels. Mean±SEM. ACTH and CORT levels were assessed under basal and stress conditions five days following the final behavioral test that occurred on day 29. **A) Males:** Following acute stress: [a]PF>C and [b] PAE>PF&C; [c] AMPH>saline exposed PAE males. **B) Females:** [#] trend for AMPH>saline exposed PAE females (p=0.03).

($p<0.04$). Further *a priori* analyses revealed enhanced ACTH following acute stress in AMPH- compared to saline-pretreated PAE, but not PF or C, males (p's<0.008).

In females, estrous stage was not a significant covariant with hormone levels ($p=0.62$). There was a stress x hormone interaction ($F_{1,100}=65.87$, $p<0.001$), and significant effects of stress ($F_{1,107}=113.21$, $p<0.001$), and hormone type ($F_{1,107}=104.64$, $p<0.001$). *Post-hoc* analyses revealed overall enhanced ACTH levels following acute stress ($p<0.001$), and a similar trend for CORT ($p=0.10$; Figure 3.4B). Further *a priori* analyses revealed a statistical trend for enhanced CORT in AMPH- compared to saline-pretreated PAE ($p=0.03$), but not C or PF, females (critical p-value=0.013 with Bonferroni correction) .

Acute stress increased testosterone (T) levels in PAE males: There was a significant prenatal group x stress interaction ($F_{2, 108}=4.77$, $p=0.01$) and a significant effect of acute restraint stress ($F_{1, 108}=14.65$, $p<0.001$). T levels were increased with acute stress in PAE ($p<0.002$) but not PF or C (p's>0.44) males (**Basal**: C: X=99.0±19 ng/mL; PF: X=74.4±9 ng/mL; PAE: X=60.8±9 ng/mL; **Stress**: C: X=116.3±14 ng/mL; PF: X=105.1±13 ng/mL; PAE: X=178.9±29 ng/mL*).

Acute stress increased progesterone (P_4) levels in females: As expected, estrous stage was a significant covariate for P_4 levels ($F_{1, 100}=10.32$, $p<0.002$). There was a significant effect of stress ($F_{1, 100}= 106.17$, $p<0.001$), with enhanced P_4 levels following acute stress (X = 99.59 ± 6.36 ng/mL) compared to basal levels (X = 28.02 ± 2.55 ng/mL) overall.

For E_2 levels, estrous stage was not a significant covariate. There was a significant effect of prenatal group ($F_{2, 100}=4.37$, $p<0.002$), with lower levels in C compared to PF ($p<0.003$) and a

trend compared to PAE (p=0.08) females (C: X=16.21±1.45 ng/mL; PF: X=26.35±2.99 ng/mL; PAE: X=21.89±2.27 ng/mL).

AMPH exposure increased spleen but not adrenal weights

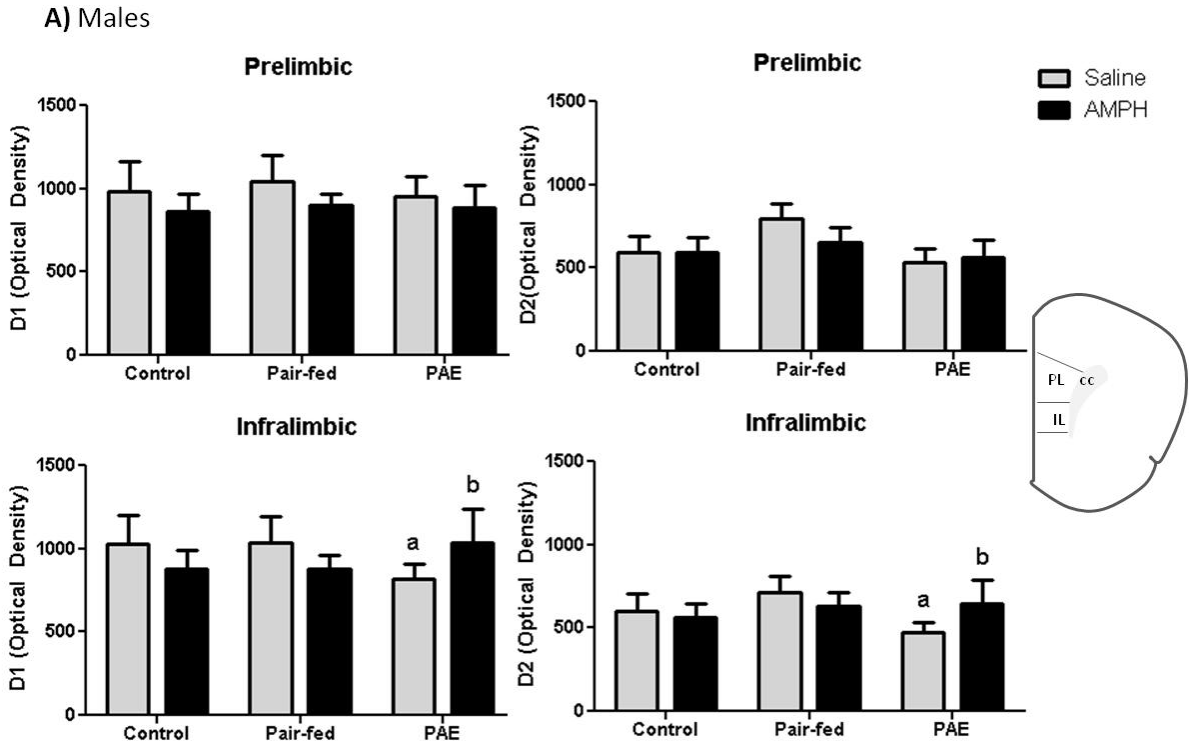
There were no significant effects of prenatal group or drug condition for either adrenal (males: p's>0.28; females: p's>0.52) or spleen (males: p's>0.15; females: p's>0.45) weights.

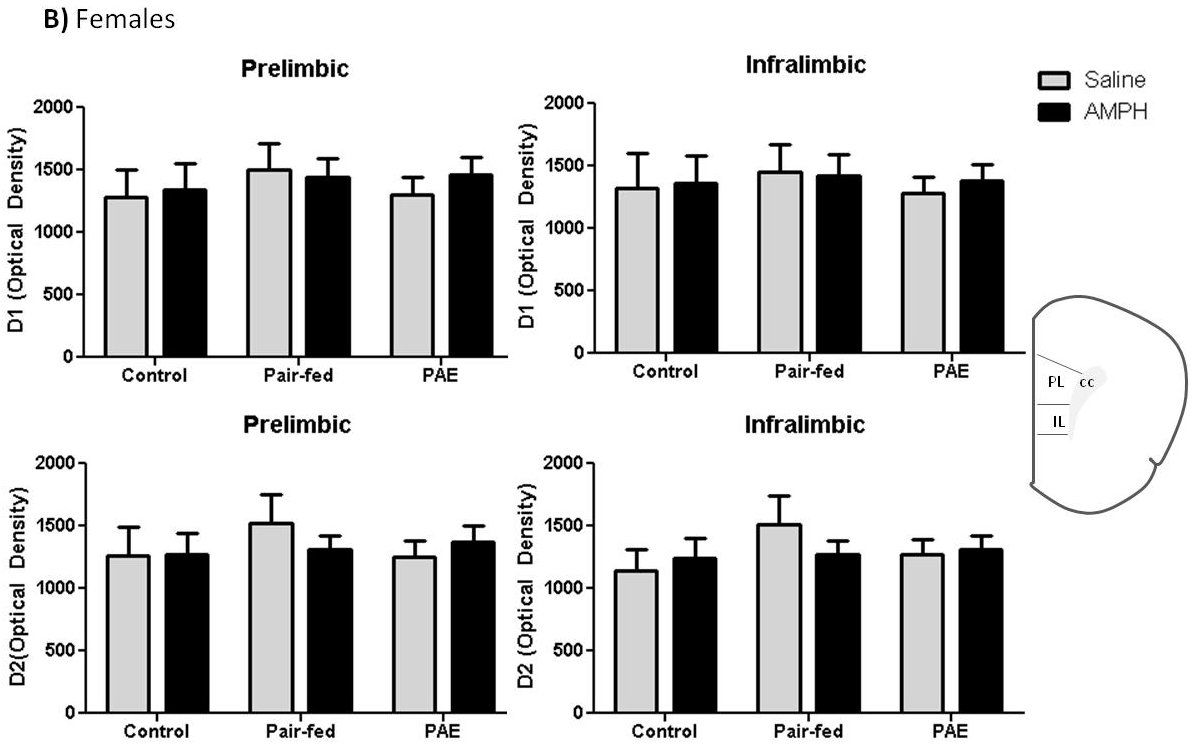
Dopamine Receptor Expression

Within the IL subregion of the mPFC, saline-pretreated PAE males displayed *increased* DA-R expression, while AMPH-pretreated PAE males displayed *decreased* DA-R expression: In males, there was a significant three-way interaction among prenatal group x drug condition x subregion interaction ($F_{2, 36}$=4.61, p=0.01), and an effect of DA-R subtype ($F_{1, 36}$=51.24, p<0.001) with more D_1 compared to D_2 expression overall (Figure 3.5A). *Post-hoc* anlayses revealed that in the IL subregion, there was *increased* expression of DA-R in saline-pretreated PAE males, but *decreased* expression in AMPH-pretreated PAE males compared to their C and PF counterparts (p's<0.001). In the PL subregion, DA-R expression in PAE males did not differ from that in C males (p's> 0.21), and was decreased compared to that in PF males following saline (p<0.001), but not AMPH (p>0.20), exposure. There was greater DA-R expression throughout the mPFC in saline-treated PF compared to saline-treated C males (p's<0.025), but no differences in AMPH-treated subjects (p's>0.21).

In females, stage of estrous cycle was not a significant covariate (p=0.91). There was a significant effect of subregion ($F_{1, 39}$=4.85, p=0.03), with greater DA-R expression in the PL

Figure 3.5. DA-R expression in the mPFC





DA-R expression in the mPFC. Mean±SEM. Levels of D_1 and D_2 are averaged in the graphs. A) **Males:** [a]Saline exposed: PAE<C=PF; [b]AMPH exposed: PAE>C & PF. B) **Females:** No differences among prenatal groups or between drug conditions.

compared to IL subregion overall (Figure 3.5B) but no other significant main or interaction effects.

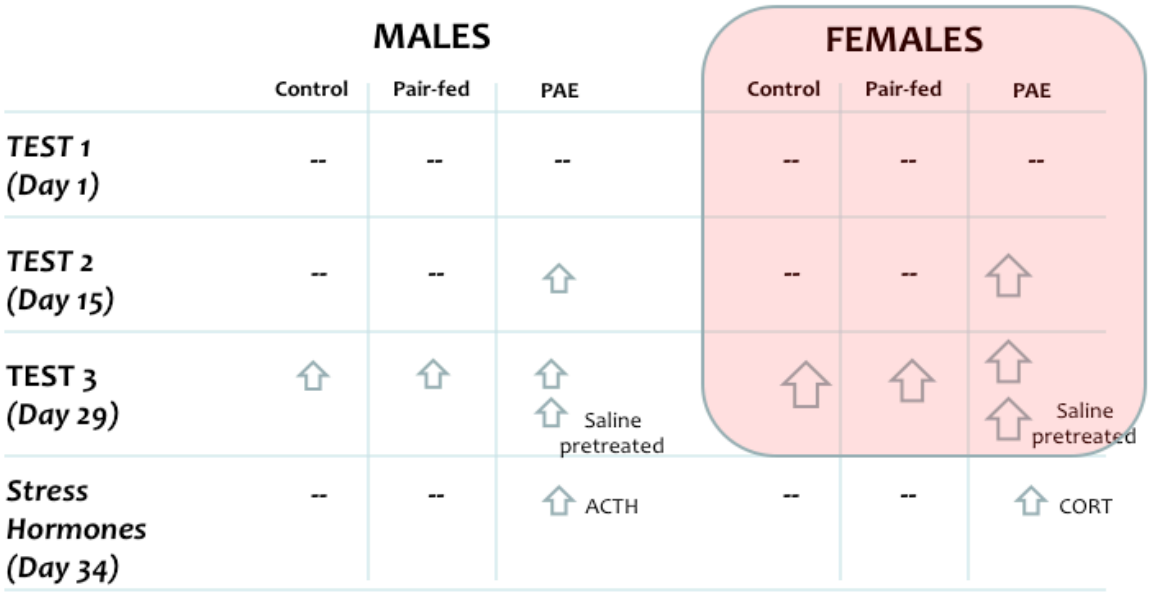
No differences in DA-R expression among prenatal groups or between drug conditions within the NAc and striatum: There were significant effects of NAc subregion and DA-R subtype in both males ($F_{1, 39}$=6.82, p=0.01 and $F_{1, 39}$=209.97, p<0.001, respectively) and females ($F_{1, 43}$=34.86, p<0.001 and $F_{1, 43}$=259.92, p<0.001, respectively). Overall, there was greater DA-R expression in the NAc shell compared to core, and overall more D_1 compared to D_2 expression (data not shown). For females, stage of estrous cycle was not a significant covariate for DA-R expression (p=0.88), and there were no other main effects or interactions.

Similarly, for the striatum, there were main effects of DA-R subtype in both males ($F_{1, 39}$=249.93, p<0.001) and females ($F_{1,43}$=202.03, p<0.001), with more D_1 compared to D_2 expression overall (data not shown). For females, stage of estrous cycle was not a significant covariate (p=0.44), and there were no other significant main effects or interactions for either males (p's>0.46) or females (p's>0.23).

3.4 Discussion

The present data demonstrate for the first time that PAE increases sensitivity to both single and repeated AMPH exposure, and alters interactions between dopamine and HPA systems (for summary see Table 3.2). I found: 1) More rapid development of behavioral activation in PAE than in control males and females; 2) Greater behavioral response to first time

Table 3.2 Summary of behavioral effects following AMPH exposure



Behavioural activation following AMPH exposure. Main findings on locomotor activation following AMPH exposure and cross-sensitization with acute stress in PAE males and females are represented.

AMPH exposure in saline-pretreated PAE but not control males and females after the mild stress of repeated saline injections; 3) Cross-sensitization between AMPH and stress in PAE but not control males and females; 4) Increased rearing in PAE, but not C and PF, males in response to AMPH injection on day 29 of testing; 5) More atypical (rotation) behavior in PAE males and females compared to their C and PF counterparts following repeated AMPH exposure; 6) Enhanced ACTH release following acute restraint stress in AMPH-treated PAE males and a similar trend for enhanced CORT release in AMPH-treated PAE females; 7) Enhanced testosterone levels following acute stress in PAE, but not control males; 8) Increased DA-R expression in the IL subregion of saline-pretreated, but decreased DA-R expression in AMPH-pretreated, PAE compared to control males. Overall, these results indicate that PAE produces enhanced sensitivity of underlying stress and dopamine systems as well as altered cross talk between stress and dopamine systems following AMPH exposure. These findings support and extend the hypothesis that neurobiological mechanisms mediating the interaction between stimulant exposure and stress are altered by PAE, in a direction supporting a neurobiological phenotype of increased vulnerability to SUDs.

Enhanced levels of 'typical' behaviors in AMPH-pretreated PAE subjects

AMPH exposure induced greater locomotor behavior than saline exposure in all prenatal groups on day 1 of injection. However, enhanced behavioral activation (i.e. greater distances travelled) occurred in PAE males and females on day 15 of testing, but not until day 29 in PF and C subjects, indicating more rapid onset of behavioral activation in PAE subjects. Additionally, sensitization (the progressive amplification of a behavioral response alongside repeated AMPH exposure) was only observed in PAE subjects, and to a greater extent in PAE females. The low

doses of AMPH did not produce significant sensitization in C and PF rats, indicating a reduced threshold for sensitization to occur in PAE males and females. AMPH-treated PAE, but not C and PF, males also showed increased rearing (another behavioral measure of activation) in response to AMPH injection on day 29 of testing. Locomotion (i.e. distance travelled) and rearing are both typical behaviors related to enhanced activity of mesocorticolimbic dopaminergic pathways, particularly within the NAc (Ikemoto, 2002). Thus, AMPH exposure may sensitize dopamine systems more rapidly in PAE, compared to C and PF, subjects. Past studies have shown earlier onset of sensitization (Vanderschuren and Pierce, 2010) and enhanced locomotor sensitization in PAE rats, as assessed by similar horizontal and vertical movements (Barbier et al., 2009), following repeated exposure to cocaine. Importantly, there are overlapping neurobiological processes that underlie sensitization of dopamine systems with different types of stimulants, as well as unique neurobiological processes specific to each type of stimulant and/or duration of stimulant exposure (Vanderschuren and Pierce, 2010). Together with our present data, it appears that PAE accelerates and enhances sensitization to stimulants, suggesting increased sensitivity of the mesocorticolimbic dopaminergic pathways under multiple drug exposure conditions.

Interestingly, following repeated saline injection (days 1-15), PAE males and females exhibited enhanced locomotion compared to controls on their first exposure to AMPH (day 29). The mild stress of repeated i.p. saline injection and exposure to novel contextual cues may have differentially sensitized dopamine systems in PAE compared to control rats, therefore facilitating the enhanced behavioral activation observed in drug naïve PAE males and females on day 29, which was not observed in their PAE counterparts that received AMPH on day 1. The finding of, attenuated weight gain throughout the experiment in PAE males and females supports the

possibility that PAE enhances sensitivity to stress. Indeed, other prenatal manipulations, such as maternal deprivation, is known to enhance behavioral responsivity to cocaine and tail-pinch stress in adult control offspring (Brake et al., 2004). Past studies have clearly demonstrated that PAE has both direct and indirect (via maternal HPA activation) effects on the developing fetal HPA axis, which reprograms fetal HPA tone and enhances sensitivity of stress systems (reviewed in (Weinberg et al., 2008).

Enhanced levels 'atypical' behaviors in AMPH-pretreated PAE subjects.

Repeated AMPH exposure enhanced rotation behavior in PAE compared to C and PF rats. Rotational behavior is considered atypical and is generally produced by exposure to dopamine agonists, including AMPH (Glick et al., 1976). Increased rotational behavior is positively correlated with increased asymmetry in nigrostriatal dopaminergic function (Glick et al., 1976, Jerussi and Glick, 1976). Specifically, asymmetry of dopamine function within the mPFC and NAc is related to either elevated dopamine levels, enhanced DA-R expression, or enhanced sensitivity of receptors, and has been positively correlated with enhanced voluntary alcohol consumption and negatively correlated with ability to cope with uncontrollable stress in control male rats (Carlson et al., 1991, 1993, Nielsen et al., 1999). In the present study, AMPH-pretreated PAE females exhibited enhanced rotation behavior compared to C and PF females on days 15 and 29, while PAE males exhibited enhanced rotation behavior compared to C and PF males throughout the duration of the experiment. These results suggest the possibility that PAE facilitates asymmetrical nigrostriatal dopaminergic function, specifically within mPFC-NAc neural loops; however, future research is needed to determine differences in DA function between the left and right hemispheres of PAE subjects. Interestingly, saline-pretreated PAE

males and females exhibited enhanced rotation behavior on the final test day (day 29) compared to their C and PF counterparts following their first AMPH exposure. It is possible that the mild stress of repeated saline injection sensitized dopamine systems to subsequent AMPH exposure. In PAE males, but not females, rotational behavior was already elevated by AMPH on day 1 of testing. Thus, PAE males appear to have an endogenous sensitivity to AMPH-induced rotational behavior.

Relationships between dopamine levels and behavior

With the protocol used in the present study, I did not demonstrate differences in severe stereotypy among prenatal groups. However I did see interesting sex differences, as males overall showed lower levels of stereotypy in the mid-range of scores (4-6) while females showed higher levels of stereotypy overall (scores 6-8). Intriguingly I did not observe any changes in DA-R levels in the striatum or nucleus accumbens among prenatal groups or following drug exposure. However it should be noted that brains were examined only at termination, 5 days after the last drug exposure and it is possible I would have observed differences at an earlier time point. AMPH binds to the dopamine active transporter and can reverse function of this protein, leading to elevated levels of dopamine and producing dose-dependent effects on motor function. As a result, a curvilinear relationship exists between locomotor activity and dopamine levels, with dopamine levels being positively correlated with overall movement. However, extremely high dopamine levels produce severe stereotypy, which is characterized by restrictive atypical body movements, and would therefore appear as *reduced* locomotion and rearing. Rotational behavior is atypical and marks the transition from exaggerated but typical behaviors to atypical behaviors, and eventually more severe stereotypy. Our present results demonstrate that there are

no differences in severe stereotypy among prenatal groups for males, but there are slight and selective increases in stereotypy in PF compared to control and PAE females. Furthermore, AMPH-treated females exhibited enhanced stereotypy overall compared to males on all three test days (1, 15 and 29), and saline-pretreated females showed enhanced stereotypy on day 29 following first-time AMPH exposure. These findings parallel past studies demonstrating enhanced behavioral sensitivity to acute and repeated AMPH exposure in females compared to males (Becker et al., 2001). In summary, PAE appears to have enhanced dopaminergic sensitivity to a level that correlated with enhanced activation of typical behaviors, as well as moderate levels of atypical behaviors, but not to levels elevated enough to produce severe stereotypies overall. This validates our findings of enhanced behavioral activation, as restrictive stereotypies did not confound the current locomotor results.

PAE facilitated cross-sensitization between AMPH and stress

In the present study, cross-sensitization between AMPH and stress was observed in the ACTH response of PAE males, with a similar trend in the CORT response of PAE females. Consistent with past findings that drug-stress or DA-stress interactions are altered by PAE (Blanchard et al., 1993, Nelson et al., 1983, Taylor et al., 1986, Uban et al., submitted-a), the present results demonstrate that AMPH enhances HPA sensitivity in PAE subjects, and does so in a sexually-dimorphic manner. Cross sensitization between AMPH and HPA hormones has been found in control males in response to higher doses of AMPH (2mg and 4mg) than those used in the present study (Barr et al., 2002), which may explain, at least in part, why cross-sensitization did not occur in controls in the present study. Furthermore, in Barr et al (2002), AMPH was administered in the home cage rather than in a separate environment with context-

specific cues, as was done in our paradigm. Context is a powerful factor in drug responsivity (Badiani and Robinson, 2004) and the formation of drug-cue associations. A study utilizing context-specific cocaine exposure, however, did not observe cross-sensitization between cocaine and stress in PAE males or females. Many of the parameters of this former study, including type of stressor and length of cocaine exposure, were different from those of the present study, which may underlie the different findings reported (Barbier et al., 2009).

I am the first to demonstrate context-dependent cross-sensitization between AMPH and stress. It is possible that the type of stimulant, stressor and extended length of stimulant exposure facilitated cross-sensitization for PAE subjects. Interestingly, contextual information relies heavily on the hippocampus (Eisch and Mandyam, 2004), which is a structure known to be highly susceptible to alterations following PAE in both males (Redila et al., 2006, Sliwowska et al., 2010, Uban et al., submitted-a) and females (Uban et al., 2010). The hippocampus underlies the initial formation of drug-cue associations, and then plays a role in monitoring ('gating') drug stimulated inputs from the nucleus accumbens, which depend on the previously acquired drug-cue associations (Grace et al., 2007). The effects of PAE on cross-sensitization may occur through dysregulation of the HPA axis, specifically implicating the hippocampus. Stress-induced alterations in hippocampal function typically reduce negative feedback regulation of the HPA axis (Goursaud et al., 2006), potentially resulting in persistent stress dysregulation and enhanced vulnerability to SUDs. Interestingly, augmented behavioral sensitivity was observed in saline-pretreated PAE males and females upon their first exposure to AMPH. This suggests that repeated mild stress (saline injection) may have revealed underlying sensitivity to AMPH to a greater extent in PAE than in control males and females. This effect may be due, in part, to

alterations in sensitivity of the hippocampus to stress following PAE (Sliwowska et al., 2010, Uban et al., 2010).

Gonadal hormones showed alterations in PAE offspring but were unaltered by AMPH exposure

Acute stress (day 34 of testing) increased testosterone levels in PAE males regardless of drug condition. Testosterone and CORT interact at multiple levels of the HPA and HPG axes, with testosterone and CORT typically playing mutual inhibitory roles (Viau, 2002). PAE may reduce basal levels of testosterone and reduces sensitivity of the HPA axis to the regulatory effects of testosterone in male rats (Lan et al., 2009, Uban et al., submitted-a). The present results extend our understanding of PAE effects on HPG function; low basal but high acute stress levels of testosterone, may contribute to the reduced capacity of testosterone to regulate HPA function in PAE males. In females, progesterone functions as a glucocorticoid antagonist via several mechanisms, mostly involving glucocorticoid receptor function (reviewed in (Kudielka and Kirschbaum, 2005). The present results demonstrate enhanced progesterone levels following acute stress in females, regardless of prenatal group or drug condition, which is possibly a compensatory mechanism involved in negative feedback regulation of the HPA axis. Together, the present results suggest that interactions between the HPG and HPA axes are unaltered by the regimen of AMPH exposure, as no differences were observed between drug conditions.

PAE and AMPH exposure interact to alter dopamine receptor expression within the infralimbic subregion of the mPFC

In the present study I found no alternations in DA-R in the nucleus accumbens and striatum, but found differential effects of prenatal group in saline- and AMPH-treated subjects.

Originating from the striatum, dopaminergic neural loops return to the striatum via the infralimbic subregion, while other projections reach the NA shell via the prelimbic subregion (Berendse et al., 1992, Ikemoto, 2007, Zahm, 1999). Once a drug-cue association has been formed with the hippocampus, the PFC is implicated in moderating drug-seeking behaviors, as well as behavioral sensitization (Andersen and Teicher, 2009, Brake et al., 2000). Our finding of reduced DA-R expression within the IL subregion of the mPFC in PAE compared to C and PF males suggests highly specific effects of PAE on dopamine function in male subjects. The finding that these alterations were reversed by AMPH exposure suggests enhanced plasticity of specific dopaminergic loops by drug exposure in the PAE brain. Repeated exposure to stimulants is known to produce a remarkable degree of neuroplasticity in underlying dopamine systems (Castner and Williams, 2007), and overall, the present results suggest that PAE has small but significant effects on stimulant-induced plasticity of these dopamine systems compared to that in controls. The drug-induced plasticity of DA-R expression in PAE males occurred in a region known to moderate dopamine release from the NAc, and ultimately influence drug-seeking behavior and behavioral sensitization (Brake et al., 2000). However, unlike males there were no significant differences observed among prenatal groups in females, on DA-R expression. Previously, I found that PAE did not alter baseline levels of DA-R expression in the mPFC, NAc or striatum in either males or females, but attenuated the typical stress-induced decrease in DA-R expression observed in control males and females in the NAc and striatum (Uban et al., submitted-a). In the present study, there were no significant differences in DA-R expression in the NAc or striatum in PAE males and females, supporting past findings that PAE does not produce differences in baseline DA-R expression within these regions (Uban et al., submitted-a). Thus, it appears that enhanced behavioral activation in PAE rats occurs without parallel changes

in DA-R expression in the NAc and striatum, indicating that other mechanisms, such as alterations in underlying plasticity, may mediate enhanced sensitivity of dopamine systems. Future studies are needed to determine differences between drug conditions and among prenatal groups for binding capacity, as well as location of DA-Rs. For example, drug-exposure can produce a bias for DA-R expression on glutamatergic neurons within motivational/reinforcement neurocircuitries projecting to the NAc (Andersen and Teicher, 2009, Kalivas et al., 2005). Thus, further investigation is needed to elucidate the relationship between behavioral sensitization and DA-R expression following PAE.

PAE alterations are consistent with a neurobiological phenotype of enhanced vulnerability to substance use disorders.

In the current study I saw that repeated amphetamine exposure enhanced typical and atypical behavioral activation to a greater extent and at an earlier time point in PAE compared to control males and females. Behavioral sensitization is known to be associated with increased addictive-like behaviors (Robinson and Berridge, 2008), and rotational behavior is indicative of asymmetrical dopaminergic function and known to be associated with mental health problems (Robinson, 1984). Stimulant sensitization paradigms are useful for modeling particular aspects of SUDs (for review see (Vanderschuren and Pierce, 2010), such as underlying neural adaptations involved in the initial development of SUDs, as well as vulnerability to relapse following prolonged abstinence (Castner and Williams, 2007). Sensitization can be observed in especially sensitive subjects after just a few drug exposures, and context-dependent sensitization tends to occur more quickly and produce longer lasting neuroplastic effects (Pierce and Kalivas, 1997). Our novel results support and extend previous knowledge of how PAE alters

neurocircuitry implicated in behavioral sensitization, which includes the mesocorticolimbic and nigrostriatal dopaminergic pathways, as well as potential alterations in drug-context associations within the hippocampus. Indeed, PAE alters dopamine function within regions implicated in these neural pathways (Barbier et al., 2009, Shen et al., 2007, Uban et al., submitted-a). Together, the present results support the hypothesis that PAE results in a neurobiological phenotype consistent with enhanced vulnerability to developing SUDs. PAE alterations in dopamine systems, as well as cross-talk with stress systems, likely play a significant role in producing neurobiological vulnerability to SUDs in both males and females. Importantly, gross differences in dopamine-sensitive behaviors and DA-R expression are not observed among prenatal groups in saline-pretreated rats. As well, endogenous sensitivity to AMPH was not observed in PAE rats, but repeated injection stress augmented sensitivity to first-time AMPH exposure in saline-pretreated PAE rats. Therefore substance exposure (in the present study) or stress exposure (Uban et al., submitted-a) appears to be required to unmask significant dysregulation of neural systems in adult PAE subjects.

Conclusions

In summary, the present data demonstrate that PAE enhances sensitivity of stress and dopamine systems, and facilitates cross-sensitization between AMPH and stress. PAE reduced the threshold for AMPH sensitization to occur, as C and PF animals did not exhibit sensitization with the low doses of AMPH exposure. These findings extend our understanding of PAE effects on the cross-talk between dopamine and stress systems, and provide insight into possible mechanisms underlying a range of psychopathologies related to dopamine and stress systems, including SUDs, depression, anxiety, schizophrenia, bipolar disorder, and neurodegenerative

disorders; all of which have a higher prevalence among individuals with an FASD than the general public (O'Connor and Paley, 2009). Importantly, an optimal level of dopaminergic function is required for executive function, learning and memory, and emotional regulation (Goto et al., 2007), all of which are altered in PAE (Mattson et al., 2011, Schneider et al., 2011). The current results support the hypothesis that PAE alters sensitivity of dopamine systems in a manner consistent with enhanced susceptibility to developing SUDs. Additionally, the current results highlight the complexity of the effects of PAE on underlying dopamine and stress systems, which warrants further investigation. An understanding of how PAE alters the neurobiological mechanisms implicated in mental health problems is vitally important for the development of specialized prevention, intervention and treatment for this unique population.

Chapter 4: Thesis summary and conclusions

4.1 Summary and implications of main findings

The current results from this thesis demonstrate that PAE alters interactions between stress and dopamine systems in a manner consistent with a neurobiological phenotype of increased vulnerability to substance use disorders. Specifically, two complementary approaches were utilized to elucidate the bi-directional interaction between stress and dopamine systems in PAE subjects: both *stress-induced* and *drug-induced* changes in basal regulation of stress and dopamine systems were examined. In **Chapter 2** the effects of chronic variable stress on basal regulation of stress and dopamine systems were examined in PAE females and males, and I found that CVS unmasked alterations in central HPA circuitry and a loss in the normal plasticity of dopamine systems in PAE compared to control animals under basal conditions. Additionally, alterations occurred in central regulation in the absence of changes in basal CORT levels in PAE males and females. PAE increased sensitivity to stress, and altered interactions between HPA and DA systems, as shown by differential effects of CVS on: 1) body weights of PAE compared to control females; 2) patterns of basal CORT over the course of CVS in PAE compared to control females; 3) downregulation of basal CRH mRNA levels in the mPFC and BNST of PAE compared to control females; 4) upregulation of basal MR mRNA levels throughout the hippocampus of PAE males and females compared to their control counterparts; and 5) downregulation of basal DA-R expression in the nucleus accumbens and striatum of control, but not PAE, animals. Overall, these results extend our understanding of the sexually-dimorphic effects of PAE on basal HPA regulation, and show, for the first time, that plasticity of *basal* HPA

and DA systems is altered differentially in males and females by PAE and CVS. In **Chapter 3**, I found that PAE increases sensitivity to both single and repeated AMPH exposure, and alters interactions between dopamine and HPA systems in adult rats. Specifically, I found: 1) sensitization in PAE males and females only; 2) greater behavioral response to first time AMPH exposure in saline-pretreated PAE, but not control, males and females after the mild stress of repeated saline injections; 3) cross-sensitization between AMPH and stress in PAE, but not control, males and females; 4) increased rearing in PAE, but not C and PF, males in response to AMPH injection on day 29 of testing; 5) more atypical (rotation) behavior in PAE males and females compared to their C and PF counterparts following repeated AMPH exposure; 6) enhanced ACTH release following acute restraint stress in AMPH-treated PAE males and a similar trend for enhanced CORT release in AMPH-treated PAE females; 7) enhanced testosterone levels following acute stress in PAE, but not control, males; 8) increased DA-R expression in the IL subregion of saline-pretreated, but decreased DA-R expression in AMPH-pretreated, PAE compared to control males under basal conditions. Overall, these results indicate that PAE produces enhanced sensitivity of underlying stress and dopamine systems as well as altered cross talk between stress and dopamine systems following AMPH exposure. These findings support and extend the hypothesis that neurobiological mechanisms mediating the interaction between stimulant exposure and stress are altered by PAE, in a direction supporting a neurobiological phenotype of increased vulnerability to SUDs.

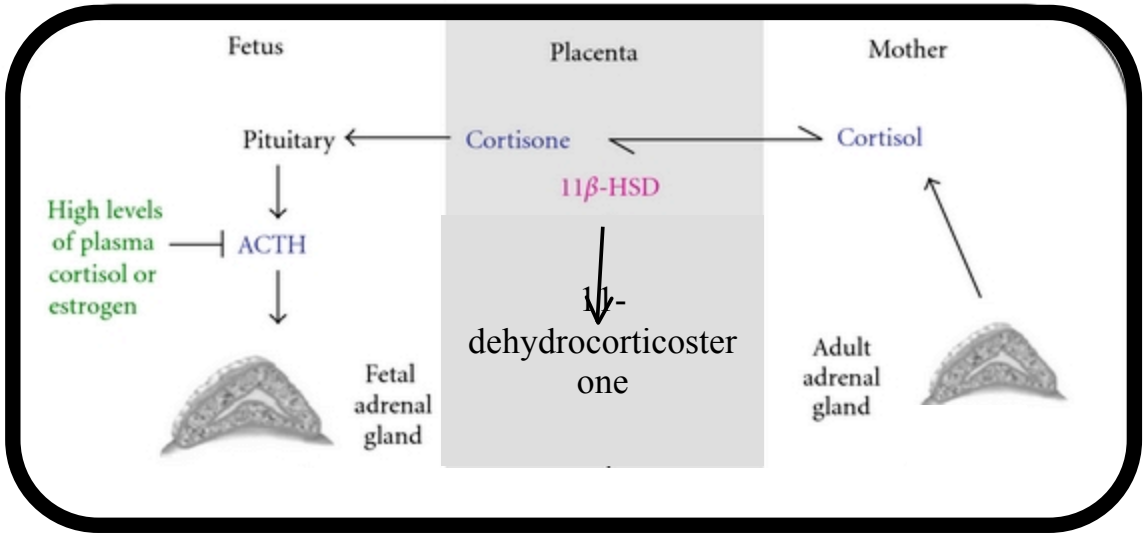
4.2 Fetal programming via PAE

Overall, the present results demonstrate developmental origins of dysregulation of adult neural systems that are relevant for vulnerability to substance use disorders. The theoretical frameworks of fetal programming and the diathesis-stress framework fit within the larger concept of DOHAD (developmental origins of health and disease). Importantly, the present results support and benefit from both theoretical frameworks. For example, the present results support the overarching theory of DOHAD, as they demonstrate that developmental factors can lead to long-lasting alterations well into adulthood. Specifically, PAE produced alterations in the effects of chronic stress on basal function, and altered effects of AMPH on activated function of both dopamine and stress systems in adult males and females. Additionally, it is more difficult to find alterations of stress systems under basal conditions, as this implies significant alterations in basal regulation of central stress systems and potentially altered homeostatic 'set points'. The concepts of fetal programming and diathesis-stress benefit the present findings, as they point to stress systems as key mediators in how the prenatal environment produces long-lasting alterations in a wide range of neuroendocrine systems and informs our understanding of potential underlying mechanisms of the observed effects. Indeed, perhaps the most widely utilized hypothesis regarding potential mechanisms by which prenatal alcohol exerts its adverse effects during fetal development is via alterations in stress systems (Ramadoss et al., 2008, Weinberg et al., 2008, Zhang et al., 2005): a finding consistently replicated in animal models and in the present results.

Fetal programming framework and FASD: Following PAE, alterations in stress and dopamine function can be observed in early postnatal life (Haley et al., 2006, Hannigan, 1990, Jacobson et al., 1999, Lee et al., 1990, Ramsay et al., 1996), and well into adulthood as observed in the present studies and others (Barbier et al., 2009, Hellemans et al., 2010b, Uban et al., 2010). Fetal programming of the HPA axis may provide a final common pathway through which a variety of different early life insults may exert their adverse long-term effects. There are several pieces of evidence that alterations in stress regulation mediate effects of PAE in the present dissertation, including enhanced sensitivity to the effects of chronic variable stress on body weight reduction, a blunted basal CORT response to chronic variable stress, and altered MR mRNA baseline levels in the hippocampus following chronic stress compared to control subjects.

Acute alcohol consumption in adulthood increases ACTH and CORT, while chronic consumption results in continued HPA activation but a dampened response to glucocorticoid challenge (Wand, 1999). During pregnancy, a time when glucocorticoid levels are naturally elevated, alcohol consumption further elevates maternal basal and stress activated glucocorticoid levels, as well as increases adrenal size without altering corticosteroid-binding globulin (CBG) levels compared to dams consuming the control or pair-fed diet (Weinberg and Bezio, 1987). Thus, the developing fetal systems received "mixed" neuroendocrine signals, which produce complex alterations in stress systems. For example, alcohol consumed by the mother crosses the placenta and directly activates the fetal stress systems. At the same time, alcohol activates the maternal stress systems, resulting in maternal glucocorticoids crossing the placenta despite the presence of 11β-hydroxysteroid dehydrogenase (11β-HSD; Figure 4.1); therefore providing enhanced negative feedback on the fetal HPA axis. Thus, alcohol consumption activates, while increased maternal glucocorticoids suppress, the fetal HPA axis during development. As

Figure 4.1 Placental regulation of glucocorticoids



In the human, 11β-HSD converts active cortisol into inactive 11-dehydrocorticosterone within the placenta and fetal tissues. Thus, 11β-HSD decreases active maternal glucocorticoids as well as those produced by fetal adrenal glands. This decreases the number of active glucocorticoids within the fetal environment. This figure has been modified with permission from (Kaludjerovic and Ward, 2012).

discussed in the introduction, several factors influence PAE effects, such as dose and timing of alcohol consumption, as well as maternal nutritional status. A range of potentially competing neuroendocrine signals can produce highly complex alterations on the developing fetal HPA axis, and these complex alterations likely share common pathways with other exposed individuals while also producing alterations that are unique to the exposed individual.

According to fetal programming, the fetal environment impacts the developing fetal systems in order to better inform the offspring of their future environmental challenges, ultimately making them better adapted for their environment (Gluckman et al., 2005, Jones, 2005). However, this theory pertains to the typical range of healthy fetal development and is not fully suitable for the effects of *in utero* alcohol exposure. Alcohol is a teratogen and produces permanent harm to the fetus that does not necessarily result in enhanced adaptation to future environmental conditions. However, it is plausible that the dual activation of the developing HPA axis, by both maternal stress and alcohol, may parallel mechanisms involved in reprogramming of the HPA axis that occur with typical development of healthy individuals. Interestingly, in the present studies, baseline dysregulation of stress systems in PAE females, but not males, sometimes appears to be 'normalized' by chronic variable stress. For example, both elevated CRH mRNA levels in the anterior bed nucleus stria terminalis and reduced MR mRNA levels in the hippocampus of PAE females appear to be 'normalized' by chronic variable stress.

Additionally, past studies have demonstrated highly specific effects of early life stress in female compared to male offspring (Kapoor et al., 2006, Lingas and Matthews, 2001, Weinstock et al., 1992) similar to the present findings. For example, females exhibited increased basal and stress-induced activity of the HPA axis, while male guinea pigs exhibited reduced basal and stress-induced activity following prenatal stress (Lingas and Matthews, 2001), suggesting that

similar *in utero* environments produce sexually-dimorphic effects on stress regulation. Sexually-dimorphic outcomes of fetal programming of stress systems make theoretical sense given the different roles in reproduction and survival between sexes.

Like the effects of PAE on the HPA axis, dysregulation of dopamine systems in PAE subjects (as found in this thesis) are likely a result of both direct and indirect effects (via maternal and fetal HPA changes). For example, the teratogenic effects of alcohol itself add insult to developing dopamine systems above and beyond potential effects of co-existing stress dysregulation with PAE. Given the bi-directional interaction of stress and dopamine systems, alterations in stress systems likely produce additional dysregulation of already altered dopamine systems. In the adult, the main mechanism through which alcohol consumption increases dopaminergic activity is through activation of dopamine cell spike activity (Grace, 2000). Thus, developing fetal dopamine systems may be permanently altered by alcohol exposure *in utero* via changes in electrical properties of dopaminergic neurons. PAE effects on dopaminergic function are likely exacerbated by dysregulation of stress systems resulting from fetal programming mechanisms.

The diathesis-stress framework and FASD: One framework for thinking about the mediating role of the HPA axis for later life outcomes in individuals with FASD is the diathesis-stress hypothesis. Adverse early life experiences may sensitize or prime stress systems. In turn, a sensitized stress axis will be hyperresponsive to subsequent, even mild, stressful life events. Interestingly, the current results demonstrate few pre-existing alterations with stress signaling and dopamine systems in PAE rats. However, various challenges (e.g. chronic variable stress, repeated amphetamine exposure, injection stress and acute restraint stress) were effective at

elucidating several PAE alterations in both stress and dopamine systems under basal conditions, implicating the diathesis-stress framework. In the present studies, several alterations in stress and dopamine systems were not observed until PAE subjects were exposed to stress. Similarly to stress system alterations, dopamine system alterations in PAE rats were not observed until PAE females and males were exposed to repeated amphetamine or mild stress of repeated saline injections. Thus, it is not simply stress that results in problems, but rather, stressors acting on an already compromised stress system. Indeed, Bita Moghaddam (Moghaddam, 2002) argues that contrary to popular theories, co-morbidity between stress and other neurological disorders is perpetuated by a dysfunctional stress response rather than stress *per se*. Additionally, endogenous sensitivity of dopamine systems was not observed in PAE rats on day one of amphetamine injections. However, enhanced sensitivity was observed in previously saline-exposed PAE rats on the final amphetamine challenge day when they received their first exposure to amphetamine, indicating that the repeated stress of saline injections was required to reveal enhanced sensitivity to first-time amphetamine exposure in PAE animals. Thus, mental pathology may result, in part, from pre-existing dysregulation of underlying neurobiological systems that increase an individual's vulnerability to a range of mental health disorders when faced with subsequent challenges such as stress and/or drug exposure. The present findings that PAE plus chronic variable stress alter basal regulation of stress systems supports the hypothesis that pre-existing differences in HPA regulation exist and can be further revealed by additional challenge.

Early life stress modulates PAE effects: The impact of early postnatal experience has received increased consideration on the final phenotype associated with FASD, as these

individuals are more likely to experience adversity in their postnatal environment. For example, individuals with FASD are more likely to be raised outside of the birth family, with a high proportion entering into the foster care system, and many experiencing instability and other stressors throughout early life including, learning and memory problems in school, and difficulties socializing with peers (O'Connor and Paley, 2009, Streissguth et al., 2004). In addition, while not always the case, those who remain in the family home may be at an increased risk of experiencing early life adversity resulting from a chaotic and unstable environment, particularly if the mother continues to drink and/or use other drugs (Mengel et al., 2006). As such, postnatal (as well as prenatal) stress may become a mediating factor in the outcome of PAE.

A prominent focus of non-human primate work has been to separate effects of prenatal stress from those of early alcohol exposure, to address the question of whether environmental factors, such as stress, augment the effects of PAE (Schneider et al., 2002). Indeed, primates prenatally exposed to alcohol plus stress show high rates of stereotypies, a behaviour indicative of vulnerability to substance use disorders, and increased behavioural activity compared to those exposed to stress or alcohol only, and compared to control primates (Schneider et al., 2001, Schneider et al., 2002). Consistent with this, in the findings from Chapter 3, I found enhanced behavioural activity was induced in PAE females and males by repeated amphetamine exposure. However, unlike primate studies investigating alcohol plus stress during the prenatal period, a high degree of stereotypies was not observed among PAE rats. Given the significant impact of maternal stress on the effects of PAE (Schneider et al., 2002), it is possible that the addition of maternal stress augments behavioural activation in alcohol-exposed offspring. Interestingly, despite different levels of observed stereotypy among prenatal groups, similar elevations in

dopamine synthesis and receptor binding were observed in the primates exposed to alcohol as levels observed in those exposed to alcohol plus stress prenatally (Schneider et al., 2005). Therefore, the addition of prenatal stress on top of prenatal alcohol exposure appears to augment behavioral responses related to dopamine function, and does so without significantly altering dopamine content itself (Schneider et al., 2002). The present findings in rats corroborate these past findings in primates, with sensitization being observed in PAE rats but without significant alterations in dopamine receptor expression. Together, these findings indicate that additional factors (such as altered endocrine regulation, alterations in another neurotransmitter system, or altered properties of dopamine receptors or enzymes themselves) are likely altered by prenatal alcohol exposure and contribute to alterations in dopamine-related behaviors.

4.3 Timeline of the developing PAE brain

Importantly, brain development is not a homogenous process. Moreover, owing to the differential maturation rate of both cortical and subcortical regions, it is likely that the impact of environmental factors will vary during different developmental stages. Maturation of specific brain regions may be required for the effects of early life adversity to appear. Differences in the time course during which early life adversity produces observable effects may be reflective of differences in rate of maturation of specific brain regions. For example, evidence suggests that although both brain regions develop over an extended period of time, the prefrontal cortex matures at a fairly steady rate, while the hippocampus undergoes relatively rapid periods of enhanced maturation throughout development (Andersen and Teicher, 2009). There are periods

throughout development where neurobiological systems are particularly sensitive to environmental factors. Moreover, the development of adult patterns of behaviour is intricately linked to the maturation of the region(s) that sub-serve the behaviour; therefore the impact of these early life experiences may not be fully appreciated or observed until later in life. In order to better understand the resulting behavioural and cognitive effects of PAE later in life, particularly sensitive developmental periods of key brain regions underlying behavioural and/or cognitive problems need to be identified, and then correlate them with: 1) timing and dose of exposure, and 2) co-occurring factors (i.e. genetics, sex, nutrition, maternal stress).

Alterations in hippocampal function following early life adversity often take longer to manifest than alterations in other regions (Andersen and Teicher, 2009). Furthermore, early life stress (pre- and post-natal) has been shown to produce significant alterations in the developing/maturing hippocampus (Andersen and Teicher, 2009, Barha et al., 2011), whereas later life adversity may be more selective for producing alterations in the prefrontal cortex (Leussis et al., 2008). Consistent with this, PAE produces significant alterations in the hippocampus such as reduced volume, altered neurogenesis and electrophysiological properties, and poor performance on hippocampal dependent tasks (Berman and Hannigan, 2000, Christie et al., 2005, Rasmussen, 2005, Redila et al., 2006, Sliwowska et al., 2010, Uban et al., 2010). The present results extend previous findings, demonstrating alterations in baseline expression of MR mRNA in PAE females, and alterations in the effects of chronic variable stress on basal MR mRNA levels in PAE males. Additionally, context-specific amphetamine sensitization involves the hippocampus (Ito and Canseliet, 2010), and the process of sensitization occurred at a lower threshold than what is required for C and PF rats. Importantly, the hippocampus is highly susceptible to PAE effects (Rasmussen, 2005, Redila et al., 2006, Sliwowska et al., 2010, Uban

et al., 2010), and given the role of the hippocampus in forming drug-cue associations (Singer et al., 2009), it is possible that these associations are enhanced in PAE rats. Interestingly, early life stress augments drug-cue associations (Andersen and Teicher, 2009), reduces baseline dopaminergic activity (Matthews and Robbins, 2003), enhances sensitivity of dopamine within the nucleus accumbens (Andersen et al., 1999, Brake et al., 2004), and results in elevated dopamine levels following cocaine exposure (Brake et al., 2004, Kosten et al., 2003). Intriguingly, these alterations in dopaminergic function following early life stress parallel changes observed in former studies on dopamine alterations following PAE (Barbier et al., 2009, Shen and Choong, 2006), further implicating stress dysregulation as a common mechanism.

Prefrontal cortex alterations are often observed shortly following an early life (but post-natal) stressor; however, prefrontal cortex alterations are not typically observed following prenatal manipulations perhaps due to its prolonged maturation (Andersen and Teicher, 2009). Interestingly, individuals with an FASD perform poorly on prefrontal cortex-dependent tasks such as executive functioning (Kodituwakku et al., 2001, Sowell et al., 2008). However, there is a gap in knowledge as to whether the prefrontal cortex is also significantly impacted by PAE in animal models. In the present studies, the postnatal environment was free from disturbances other than weekly cage changing, and this environmental stability may have served as a protective factor against prefrontal cortex impairments in PAE subjects, as alterations or delays in maturation resulting from PAE may resolve by adulthood. Overall, baseline alterations were observed within the prefrontal cortex following PAE when examined in adult females, but only following additional postnatal stress (repeated saline injections stress) or amphetamine exposure in adult PAE males.

4.4 Developmental origins of substance use disorders following PAE

Prenatal and early life experience, such as *in utero* alcohol exposure and/or stress, can have an enduring impact on neurobiological systems, including those implicated in drug reinforcement and stress (Frye et al., 2011). Alterations in HPA and dopaminergic function are further influenced by early postnatal life when these systems continue to mature (Feder et al., 2009). Underlying neurobiological mechanisms that may be susceptible to additional early life insult that influence vulnerability to substance use disorders in PAE affected individuals include: 1) dysregulation of stress systems; 2) dysregulation of dopamine systems, and 3) alterations in HPA-dopamine interactions. Together, the present results of this dissertation demonstrate that PAE alters all three of these potential mechanisms. I observed enhanced sensitivity of stress systems to chronic stress (Chapter 2; (Uban et al., submitted-a) and enhanced sensitivity of dopamine systems to repeated amphetamine exposure (Chapter 3; (Uban et al., submitted-b) in PAE rats. Further, the present results demonstrate clear alterations in the bi-directional interaction between stress and dopamine systems. For example, the effect of chronic variable stress on dopamine receptor expression was attenuated in the nucleus accumbens and striatum of PAE rats under basal conditions, and repeated amphetamine exposure augmented HPA reactivity in PAE rats.

A leading theory of addiction involves an imbalance of prefrontal to limbic regions in adults (Volkow and Fowler, 2000). Interestingly, compared to healthy adults, neural activation is reduced within the frontal cortices but enhanced within the nucleus accumbens of healthy children (Durston, 2003, Ernst et al., 2005), indicating that an imbalance between frontal to limbic function may be more characteristic of an immature neural phenotype. In support of this

hypothesis, 'adolescent' rodents more readily develop drug-cue associations (Badanich et al., 2006), and are more resistant to extinction of drug-cue associations (Brenhouse and Andersen, 2008) compared to their adult counterparts. More specifically, problematic substance use may parallel an imbalance between dopamine circuitries underlying executive functioning and decision making with those underlying substance-related reinforcement and conditioning (Everitt et al., 2008, Volkow et al., 2011). For example, dopamine responses in individuals with substance use disorders are blunted in response to substance administration, but enhanced in response to substance-conditioned stimuli in the absence of the substance itself (Volkow et al., 2011). Accumulating evidence suggests that problematic substance use is correlated with reduced executive function and emotional regulation, supporting the theory of an acquired drug-induced imbalance of dopamine systems (Di Chiara and Bassareo, 2007). Indeed, individual differences in certain cognitive functions, such as impulsivity, may be a result of inherent differences in dopamine function, possibly related to genetic polymorphisms (Le Moal, 2009, Sweitzer et al., 2012). For example, reduced binding of type 1 and 2 dopamine receptors (D_1 and D_2), as well as functional polymorphism variants of their genes, have been associated with increased susceptibility to substance use disorders (Hooks et al., 1994, Sweitzer et al., 2012, Volkow et al., 1999, Volkow et al., 2011). Reduced binding of dopamine receptors (D_1 and D_2) is also correlated with attenuated baseline activation of the prefrontal cortex (orbitofrontal cortex) in humans, but augmented activation in response to drug-conditioned stimuli compared to controls (Heinz et al., 2004, Volkow and Fowler, 2000, Volkow et al., 2011).

The prefrontal cortex is involved in monitoring 'motivational salience', by influencing nucleus accumbens outputs via excitatory inputs from the prefrontal cortex to nucleus accumbens (Kalivas et al., 2005), which are then modulated by the hippocampus. Similar to the present

findings with PAE (Chapter 3), past models of early substance or stress exposure have found facilitation of sensitization to amphetamine (Deroche et al., 1992, Doremus-Fitzwater and Spear, 2010), further supporting the hypothesis that prenatal environment produces long lasting alterations in neural systems underlying amphetamine sensitization.

In Chapter 2, I found that PAE did not alter baseline levels of dopamine receptor expression in the medial prefrontal cortex, nucleus accumbens or striatum in females or males, but attenuated the typical decrease in dopamine receptor expression following chronic variable stress that was observed in control males and females in the nucleus accumbens and striatum. There were no significant differences in dopamine receptor expression in the nucleus accumbens or striatum in PAE females and males, supporting findings that PAE does not produce differences in baseline dopamine receptor expression within these regions. In Chapter 3, I found that previously saline-exposed PAE males exhibited reduced dopamine receptor expression compared to control and PF males, but repeated amphetamine exposed PAE males exhibited enhanced dopamine receptor expression in the medial prefrontal cortex under baseline conditions. Discrepancies between findings regarding reduced dopamine receptor expression in the medial prefrontal cortex of PAE males between the two studies in this dissertation may be accounted for by the fact that receptor expression was measured after repeated saline injections (i.e. repeated stress exposure) compared to undisturbed conditions. This would suggest that PAE males are more sensitive to the effects of repeated mild stress on dopamine receptor expression within the medial prefrontal cortex. However, the effects of chronic variable stress (i.e. 10 days) on dopamine receptor expression were also investigated, and PAE *attenuated* the decrease in dopamine receptor expression following exposure to chronic variable stress in PAE rats. There were many varying factors that may account for this discrepancy between findings from both

studies (Chapter 2 and 3) given the entirely different paradigms utilized between studies. For example, the timeline for investigating baseline levels of dopamine receptor expression, as well as the types of stressors varied greatly between the two studies. Both studies investigated dopamine receptor expression under basal conditions, but expression was investigated 24 hours following the last day of chronic stress in one study, while expression was examined 19 days following the cessation of repeated injection stress. It is possible that any reduction in dopamine receptor expression that may have occurred as a result of repeated injection stress was resolved by the time dopamine receptor expression was assessed. This explanation is corroborated by the observation that repeated stress reduced dopamine receptor expression in control subjects in the study examining expression 24 hours after the last stressor, while repeated injection stress did not reduce expression in the study utilizing a 19 day washout period. Additionally, effects of stress are specific to type and duration of the stressor(s) (Lupien et al., 2009), which both differed between studies. However, there were no significant pre-existing differences in dopamine receptor expression in the nucleus accumbens or striatum in PAE males and females in both studies.

4.5 Potential mechanisms underlying PAE effects on dopamine systems

Past studies have found that PAE produces marked alterations in dopamine systems (Blanchard et al., 1993, Shen et al., 2007, Shen et al., 1999, Shetty et al., 1993, Spear, 1996, Wang et al., 2006) with overall reduced tonic activity of dopamine systems following PAE. Reduced activity is observed in young and older male rats following PAE, and is not due to a

reduction in dopamine neuron numbers, firing rates or patterns, indicating the presence of a depolarization block, rather than enhanced inhibition in PAE animals (Shen et al., 2007; Shen et al., 1999; Xu and Shen, 2001). The enhanced inhibition of tonic dopaminergic activity could result from either excessive excitation or from alternative inputs to dopamine neurons within the ventral tegmental area (Shen and Choong, 2006). Although the exact molecular and cellular consequences that maternal alcohol consumption has on the developing fetal dopamine systems are unknown, it is possible that stress hormones play a significant role. For example, chronic stress initially increases, then decreases, tonic dopamine activity in the nucleus accumbens (Cabib and Puglisi-Allegra, 2012). Reduced tonic dopamine activity in PAE rats may result, in part, from chronic activation of maternal stress systems. Another potential mechanism could be effects of alcohol exposure on developing neural mechanisms underlying synaptic plasticity. For example, activation of dopamine cell spike activity is caused by alcohol consumption, resulting in overall increased dopaminergic activity as a direct result of changes in electrical activity in the typical adult (Grace, 2000). Long-term potentiation (LTP) and depression (LTD) are essential underlying processes for enhancing and decreasing synaptic strength, and PAE alters these properties in dopamine neurons (Shen et al., 1999). For example, within the CA1 subregion of the hippocampus, the rate of decay of LTP was accelerated in PAE animals (Savage et al., 1998). A dose-dependent effect of PAE was observed, with the highest alcohol dose resulting in the greatest attenuation in the evoked spike amplitudes of neurons within the CA1 (Krahl et al., 1999). These alterations may be due, in part, to abnormal paired-pulse responses (Berman and Hannigan, 2000), which are necessary for effective LTP. Interestingly, repeated exposure to a low dose of a central nervous system stimulant (i.e. amphetamine or methylphenidate) was able to reverse the apparent depolarization block, restoring basal dopaminergic activity levels in PAE

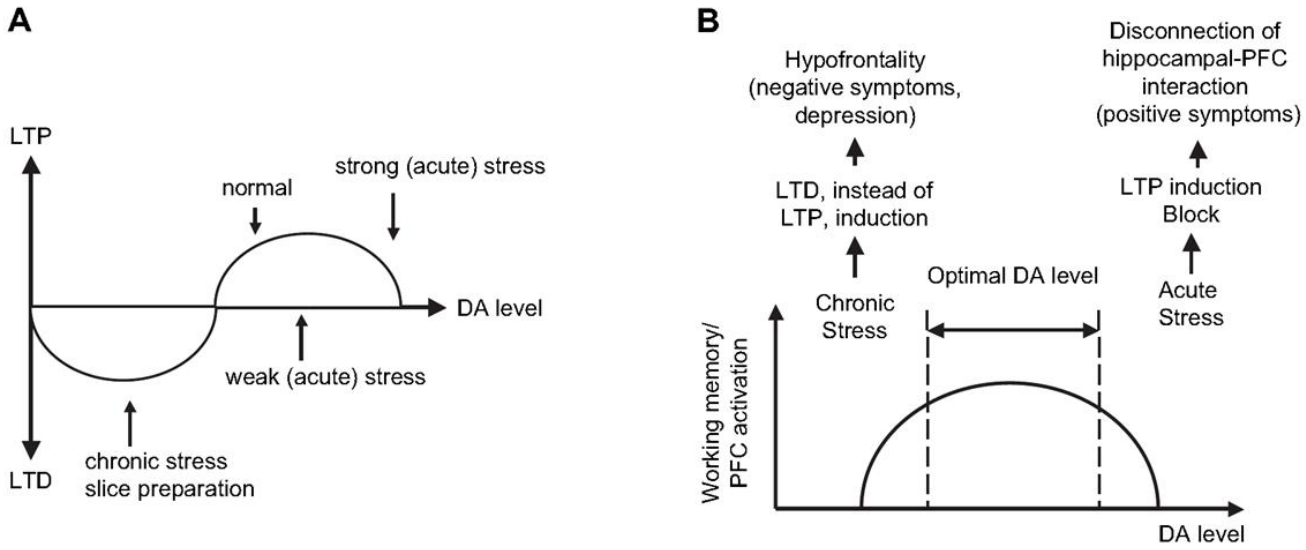
animals to levels observed in control males (Choong and Shen, 2004b; Shen and Choong, 2006; Xu and Shen, 2001). Thus, alterations in synaptic plasticity via alterations in electrical properties may underlie some the effects of PAE on reduced tonic dopamine levels, and may be a direct result of the teratogenic effects of alcohol.

Interestingly, LTP in the PFC is dependent upon dopamine levels in prefrontal neurons (Otani et al., 2003), and perhaps in other regions yet to be investigated. Within the prefrontal cortex, short duration stress increases baseline dopamine activity resulting in facilitation of LTP (Gresch et al., 1994, Morrow et al., 2000), while prolonged stress reduces baseline dopamine activity resulting in impairment of LTP (Goto and Grace, 2006, Gresch et al., 1994), and eventually a preference for LTD is observed (Goto and Grace, 2006) (Figure 4.2). Importantly, too high or too levels of dopamine within the prefrontal cortex have been linked to increased impairments in working memory (Goto and Grace, 2006). In a typical brain with a moderate level of baseline dopaminergic activity, a moderate stressor would increase dopaminergic activity to a degree associated with optimal performance, but with an intense stressor or pre-existing hypersensitivity to stress, dopamine activity may be enhanced to a very high level that is associated with impaired performance (Figure 4.2). However, in an individual with very low baseline dopaminergic activity as a result of chronic stress, and perhaps with reduced tonic activity observed in PAE rats, LTP is impaired and LTD induction becomes the predominant neurobiological response, which may be a mechanism that contributes to hypofrontality in PAE individuals and impaired working memory. Hypofrontality may be a mechanism underlying etiology of mental health problems including depression and substance use disorders, and is directly related to reduced baseline dopaminergic activity (Goto et al., 2007, Howes and Kapur, 2009). Pertaining to the present results, the attenuation of the effects of chronic stress on

dopamine receptor expression in PAE rats may result from a pre-existing reduction in tonic dopamine activity in PAE animals, resulting in impaired plasticity as measured by LTP, LTD or as in the present studies, reduced flexibility of dopamine receptor expression in response to chronic stress exposure. Further, the restoral of basal dopaminergic activity in PAE animals following repeated exposure to a low dose of amphetamine may involve restoration of plasticity including LTP induction in PAE animals (Choong and Shen, 2004b; Shen and Choong, 2006; Xu and Shen, 2001); however, future investigation is needed.

PAE alters plasticity of stress and dopamine systems: Alterations in stress-induced plasticity are observed in PAE animals in both Chapter 2 and other studies. For example, the typical neurogenic response to chronic stress in the hippocampus is altered in PAE males (Sliwowska et al., 2010) and females (Uban et al., 2010). Given alterations in plasticity of dopamine and stress systems in PAE animals as seen in Chapter 2 and 3, it is possible that alterations in HPA-dopamine interactions arise in part from altered plasticity. For example, PAE rats demonstrated increased HPA responsivity to alcohol and morphine challenges compared to control animals (Nelson et al., 1986, Taylor et al., 1986), and enhanced stress-induced alcohol consumption (Nelson et al., 1983). In the present dissertation, changes in dopamine receptor expression to chronic stress exposure was attenuated in PAE rats (Uban et al., submitted-a), and repeated amphetamine exposure resulted in facilitation of behavioural sensitization as well as cross-sensitization with acute stress in PAE rats (Uban et al., submitted-b). Together, these findings suggest enhanced sensitivity of stress systems to alterations by dopaminergic activation in PAE rats, but the effects of repeated stress on dopamine-dependent outcomes is not as clear,

Figure 4.2 Relationship between dopamine levels and cognitive performance



Relationship between dopamine levels and cognitive performance. Prolonged stress reduces baseline dopamine activity resulting in impairment of LTP, and eventually a preference for LTD is observed. Thus, an appropriate level of dopamine is needed for optimal function. Importantly, too high or too low of dopamine levels within the prefrontal cortex have been linked to increased impairments in working memory. In a typical brain with a moderate level of baseline dopamine activity, a moderate stressor would increase dopamine activity to a degree associated with optimal performance, but with an intense stressor or pre-existing hypersensitivity to stress, dopamine activity may be enhanced to very high levels associated with impaired performance. However, in an individual with very low baseline dopamine activity LTP is impaired and LTD induction becomes the predominant neurobiological response, which could lead to prolonged hypofrontality. Adapted from (Goto, Otani & Grace, 2007) with permission to reprint obtained from Neuropharmacology (License Number: 2941420449780; Type Of Use: Dissertation/Thesis).

with a *reduction* in the effects of chronic stress on dopamine receptor expression, but *enhanced* stress-induced alcohol consumption in PAE rats (Nelson et al., 1983). The present data support and extend these findings, indicating that PAE alters plasticity, with evidence for both enhanced and reduced plasticity pertaining to stress and dopamine systems.

4.6 Altered metaplasticity may influence vulnerability to substance use disorders in PAE rats

Metaplasticity refers to a persistent change in the capacity to produce typical synaptic plasticity, implementing molecular changes involving LTD and LTP (Abraham, 2008). Overall, the present findings suggest that PAE reduces metaplasticity of stress and dopamine systems, where neurobiological responses are not as finely calibrated to a range of stimuli. As a result, PAE subjects may show exaggerated or blunted plasticity of neural systems compared to control subjects. Former studies have shown reductions in plasticity following PAE (Redila et al., 2006, Rema and Ebner, 1999, Sliwowska et al., 2010, Uban et al., 2010), and the present findings extend these findings to demonstrate *reduced* flexibility of dopamine receptor expression in response to chronic stress exposure (Chapter 2), but *enhanced* amphetamine-induced plasticity of dopamine systems (Chapter 3). When trying to connect past and present findings on the effects of PAE on plasticity, the concept of metaplasticity has some utility. PAE may reduce the gradient of finely tuned neurophysiological responses of stress and dopamine systems, resulting in a mismatch between stimuli and appropriate neurobiological responses. Reduced metaplasticity in PAE subjects may result in less behavioural flexibility, which would appear as reduced or exaggerated responsivity depending on the stimulus, environmental context and an

individual's past experience. As a result of reduced metaplasticity, deficits in the ability to correctly interpret environmental stimuli as well as predict outcomes would be expected in individuals with a FASD. Evidence in support of this theory includes the high prevalence of incarceration rates, social skill problems and unemployment rates among individuals with a FASD (Brintnell et al., 2010, Streissguth and O'Malley, 2000), indicating a mismatch between interpretations of environmental stimuli with appropriate behavioural/cognitive responses.

Acquiring a substance use disorder involves dramatic changes in behaviour, indicating significant neuroadaptation. Interestingly, substance use disorders have been described as highly inflexible behaviour (Andersen and Teicher, 2009), which may result from overlearning of drug-cue associations (Hyman et al., 2006) or impairments in neuroplasticity (Kalivas and O'Brien, 2008). For example, substance use disorders involve overlearning (initial enhanced plasticity) (Eisch et al., 2008), followed by highly inflexible behaviour (reduced plasticity resulting in a strong bias for neurocircuitries supporting drug-seeking and drug-wanting behavior/cognition). Current results demonstrate *enhanced* dopaminergic plasticity with repeated exposure to amphetamine, but *reduced* dopaminergic plasticity following chronic variable stress. Together, these results support the theory that PAE produces changes in metaplasticity, not just enhances or decreases plasticity.

Interestingly, GABAergic systems facilitate behavioural flexibility, with increased GABA allowing several different sources to influence glutamatergic output (Seamans and Yang, 2004). However, during repeated substance exposure and/or chronic stress, D_1 is overexpressed on glutamatergic outputs to the nucleus accumbens (Kalivas et al., 2005), and this may underlie preferential activation of circuitries that promote addictive-like behaviours. The current results did not find significant differences in basal expression of D_1 or D_2 in PAE subjects. However, the

location of dopamine receptors on specific neurons (ie glutamatergic) was not determined in the present studies, and further investigation is needed to determine if PAE produces an increased proportion of dopamine receptors on glutamatergic neurons within motivational/reinforcement dopamine circuitries.

4.7 Significant strengths of the current research

Strengths of present research include: the investigation of multiple brain regions, rather than focusing on a single region; elucidation of the bi-directional nature of HPA-dopamine interactions; and the inclusion of both sexes in the studies. Although focus on a single region often provides highly detailed information, the assessment of numerous regions within stress and dopamine neurocircuitries better captures whole system dysregulation. Given the wide spread effects of PAE on the brain, this approach proved to be highly valuable in determining alterations throughout key brain regions. Additionally, multiple stress (ACTH, CORT) and gonadal hormone (testosterone, estradiol, progesterone) levels were assessed. Brain signaling influences hormone production in the body, and likewise, peripheral hormones provide feedback to the brain. Thus, the elucidation of alterations in hormones not only provides increased understanding of sexually-dimorphic effects, but also better captures potential alterations in brain-body interactions.

An additional strength of the current research is that the bi-directional interactions between stress and dopamine systems were investigated with different paradigms, allowing for multiple perspectives on neurobiological alterations following PAE. Each paradigm elucidates

unique aspects of the bi-directional interactions among HPA-dopamine systems. For example, chronic variable stress is a well validated paradigm that is particularly valuable for looking at stress-mediated neurobiological effects linked to mental health disorders. Chronic variable stress is known to produce behavioural aspects in rats linked to mental health problems, especially depression, anxiety and substance use disorders (Willner, 2005). The present findings demonstrated that prenatal treatment altered the pattern of basal CORT activity during chronic variable stress in females. Control females showed an elevation of basal CORT levels midway through CVS treatment, and a return to baseline levels by the end of CVS. This profile of **basal** CORT levels was attenuated in PF females and absent in PAE females, suggesting that PAE blunts the normal basal CORT response to CVS. Interestingly, basal CORT levels were unaltered in males. These results indicate sexually-dimorphic habituation of the HPA axis to repeated, uncontrollable, unpredictable and psychological stress, as well as sexually-dimorphic effects of PAE on HPA habituation. Furthermore, a wide range of alterations in basal MR mRNA, CRH mRNA and dopamine receptor expression was observed despite comparable basal CORT levels among prenatal groups. Together, this suggests a disconnect between peripheral CORT levels and measures of central stress regulation following chronic stress, and this disconnect differs among prenatal groups and between males and females overall.

In Chapter 3 I tested the effects of repeated amphetamine exposure on behavioural activation, as well as cross-sensitization to acute stress. The connection between sensitization and vulnerability to substance use disorders has been well validated such that enhanced behavioural sensitization to repeated amphetamine is associated with a greater vulnerability to addictive-like behaviors (Robinson and Berridge, 2008, Vanderschuren and Pierce, 2010). Specifically, PAE rats exhibited sensitization at a lower threshold than controls.

Reinforcement/motivational dopaminergic neurocircuitries are subject to long-term changes induced by repeated amphetamine exposure, and these changes correspond with greater behavioural responsivity to subsequent challenge by either acute amphetamine or acute stress exposure. A primary hypothesis is that enhanced sensitivity of underlying dopamine systems leads to enhanced drug-induced neuroplasticity, resulting in enhanced reinforcement of neural pathways that promote drug-seeking behaviours and learning of drug-cue associations (Koob and Kreek, 2007). The results from Chapter 3 demonstrate enhanced malleability of stress systems following repeated amphetamine exposure, and in a direction that supports a neurobiological phenotype of increased vulnerability to substance use disorders. Further, most sensitization paradigms quantify infrared beam breaks, allowing for measures of vertical and horizontal activity only. In the present study, multiple behavioural measures were examined, allowing for inspection of rotational behaviour to indicate potential asymmetry in underlying nigrostriatal dopamine systems, as well as stereotypies.

Additionally, context-specific amphetamine exposure was utilized, which enhances face validity of this paradigm. For example, individuals with substance use problems typically form an excess of drug-associated cues or 'overlearn' cues that indicate drug availability, and therefore play a significant role in the problematic substance use (Hyman et al., 2006). Context-specific amphetamine exposure allows for a larger role of the hippocampus in modulating dopaminergic activity within the nucleus accumbens when drug-cue associations are activated. Given the effects of PAE on a number of neurobiological alterations within the hippocampus (Christie et al., 2005, Sliwowska et al., 2010, Uban et al., 2010), as well as the role of the hippocampus in modulating dopaminergic activity and forming drug-cue associations (Goto et

al., 2007, Grace, 2000, Ito and Canseliet, 2010), utilizing a context-specific sensitization paradigm was a major strength of the present study.

In the current studies, although the effects of chronic variable stress or repeated amphetamine exposure often overlapped between male and female subjects, obvious sex differences in the effects of PAE on stress and dopamine effects were observed. For example, repeated amphetamine exposure produced a higher degree of stereotypies in females than males overall. Previous research has consistently demonstrated enhanced sensitivity to stimulants in females compared to males, as well as enhanced behavioural sensitization to repeated amphetamine exposure (Becker et al., 2001). Importantly, gonadal hormones play a significant role in the production of enhanced sensitivity of females, as behavioural responsiveness is greatest during estrus, and reduced by ovariectomy (reviewed in (Becker et al., 2001). Sex differences in the effects of PAE on dopamine alterations are observed as well. For example, PAE augments alcohol-induced increases of dopamine in the nucleus accumbens and striatum in males, but did not alter dopamine release in the nucleus accumbens and attenuated release in the striatum of PAE females (Blanchard et al., 1993). Together, this suggests potentially different dopaminergic circuitries and function in males compared to females overall. With half of the world's population being female, the inclusion of females in all levels of research should be a standard expectation. Findings in females overall are in line with past research findings indicative of an enhanced vulnerability to mental health problems compared to males. Enhanced neurobiological vulnerability observed in female subjects overall may be related to overall increased basal HPA tone, and an often elevated stress responsivity compared to males. However, the inclusion of both sexes is still not a standard for pre-clinical research, but the need

to include both sexes is becoming increasingly recognized and implemented, and is further advocated by the current findings.

The current dissertation also measured gonadal hormones, and investigated stage of estrus as a covariate in statistical analyses; and is therefore a more thorough investigation of sexually-dimorphic effects. For example, PAE males exhibited reduced basal testosterone levels, but enhanced testosterone levels following acute restraint stress in the present studies. The current findings support past findings that PAE reduces sensitivity of the HPA axis to the regulatory effects of testosterone (Lan et al., 2009), indicating HPG dysregulation in PAE males. Testosterone interacts with CORT at multiple levels of the HPA and HPG axes, with testosterone typically playing an inhibitory role on HPA activity (Viau, 2002). In PAE males, low testosterone levels could diminish the capacity to regulate HPA activity and contribute to the HPA hyperresponsiveness that is typically observed. In females, there were no effects of prenatal treatment or stress on estradiol levels. However, reduced basal levels following chronic variable stress, but enhanced progesterone levels following acute stress were observed in females regardless of prenatal group. Progesterone functions as a glucocorticoid antagonist on the HPA axis via several mechanisms, primarily involving glucocorticoid receptors (reviewed in (Kudielka and Kirschbaum, 2005); therefore the present findings likely reflect a compensatory mechanism involved in negative feedback of progesterone to the HPA axis. Furthermore, it is possible that decreased basal progesterone levels following chronic variable stress may result in reduced antagonism of HPA activity, and may provide one mechanism through which stress increases overall vulnerability to subsequent stressors in females. Current results indicate that interactions between the HPG and HPA-axes are unaltered by the regimen of repeated amphetamine exposure as no significant differences were observed between drug conditions

(Chapter 3). Overall, alterations in HPG function in PAE subjects further demonstrates the complexity when considering the effects of PAE on stress dysreguation, as stress systems do not operate in isolation but rather interact with additional dysregulated systems.

Pair-feeding is an experimental treatment in itself: A number of effects of pair-feeding were observed in the present studies. The pair-feeding condition acts as a control for the reduced nutritional intake of the PAE dams, and although alcohol consuming dams eat *ad libitum*, they typically reduce their intake below what would occur if given the same diet without alcohol, and the volume of diet for PF dams is yoked to that of PAE dams. Thus, PF dams are effectively on a "meal feeding" schedule. They get less food than they would eat *ad libitum*, and often consume their entire ration within a few hours, remaining deprived until the next feeding. It is likely that PF dams experience some level of stress as a result of potential hunger that accompanies reduced food intake, and in turn, their offspring may be exposed to prenatal stress. Given the significant role of prenatal stress on dysregulation of stress systems, as well as drug-cue associations and enhanced sensitivity to drug exposure, PF offspring serve as a unique treatment group in itself for investigating vulnerability to substance use disorders. Thus, the inclusion of a pair-feeding treatment group serves as an additional strength in the present dissertation.

Indeed, unique effects were observed in the present studies in PF offspring. In contrast to PAE females who showed *decreased* CRH mRNA levels in the prelimbic subregion of the medial prefrontal cortex, PF females showed *increased* CRH mRNA levels in the infralimbic subregion (Chapter 2). Chronic variable stress altered CRH mRNA throughout the bed nucleus of the stria terminalis in PAE females, and in the posterior bed nucleus of the stria terminalis in

control females, while there were no significant effects in PF females. While control males exhibited decreased D_1 and D_2 expression in the nucleus accumbens and striatum, PF males exhibited the same decrease in D_1 but not D_2, suggesting that pair-feeding and chronic variable stress interact to selectively alter D_1 in males. Like PAE females, PF females showed an attenuation of the typical decrease in dopamine receptor expression in response to chronic stress exposure in the nucleus accumbens, but, intriguingly, an increase in the striatum for both D_1 and D_2 receptor subtypes suggesting that pair-feeding and chronic variable stress interact to produce dopamine alterations in the opposite direction of control females. Pair-feeding also altered the interaction between chronic variable stress and dopamine receptor expression in a sexually-dimorphic and region-specific manner different from that observed in PAE rats. Interestingly, PF females and males exhibited similar levels of behavioural activation to repeated amphetamine exposure as those of control counterparts (Chapter 3). However, PF females exhibited the greatest degree of stereotypy compared to both PAE and control females following repeated amphetamine exposure, indicating that pair-feeding may produce greater effects on sensitivity of dopamine systems in female offspring. Overall, given the potential stress experienced by PF dams as well as reduced nutrient intake, it is not entirely surprising that some effects of pair-feeding are observed in the present studies and that some effects are unique from those demonstrated by PAE subjects.

4.8 Limitations of the current dissertation research

The model of prenatal alcohol exposure that was utilized in the present dissertation has several strengths but also some limitations. One limitation is the lack of alcohol exposure during

the third trimester human equivalent (which occurs during the postnatal period in the rat). The third trimester is a period during which a large degree of central nervous system development occurs (Avishai-Eliner et al., 2002). Although our paradigm does not include alcohol exposure during this 'opportunistic' time period to produce alcohol-exposure related effects, I was still able to demonstrate significant alterations in basal regulation of stress systems, and alterations in dopamine systems. Furthermore, the lack of third trimester human equivalent alcohol exposure in our rat model of prenatal alcohol exposure does provide the model some face validity as pregnant women who drink during pregnancy typically reduce or eliminate intake during the third trimester (Ebrahim and Gfroerer, 2003).

Within the present results of this dissertation, there is a lack of information about PAE alterations on circadian rhythms of CORT secretion. Given the significant link between flattened or exaggerated circadian rhythms of CORT secretion with stress-related disorders including substance use disorders (Lovallo, 2006), this would be highly valuable information for understanding PAE alterations on stress systems. For example, naloxone blocks opioid input to CRH neurons within the hypothalamus, resulting in enhanced CORT secretion, and can therefore be used to measure opioid mediation of HPA responsivity in humans and provide insight into alterations in HPA circadian patterns. In humans, non-alcohol dependent individuals with a positive family history of alcohol dependence (one or both parents) exhibited maximal HPA sensitivity to a low dose of naloxone (an opioid inverse agonist), whereas individuals with a negative family history exhibited a gradient of dose-dependent HPA responses (Wand et al., 2001, Wand et al., 1998). This indicates that a positive family history may result in diminished hypothalamic opioid activity, resulting in a loss of the typical gradient in HPA responsivity. Although individuals with a FASD have not been investigated for alterations in circadian

patterns of HPA activity or for opioid dysregulation of HPA regulation, there is a wealth of evidence supporting pre-existing alterations in HPA function including in the present studies, which together demonstrate a similar loss of the gradient of HPA responses to varying levels of stressful stimuli (reviewed in (Weinberg et al., 2008).

An additional limitation of the present dissertation is the lack of information of altered HPA regulation under an activated state. Although investigation of basal regulation of stress systems is highly valuable and often understudied, the combination of investigating both basal and stress-activated alterations would be highly valuable. Understanding how basal and stress-activated regulation of stress systems are altered within the same subject would provide a more comprehensive picture of the effects of prenatal alcohol exposure on stress systems. There are obvious methodological limitations to looking at regulation of central stress systems within the same subject if performing immunohisotchemistry or *in situ* hybridization to assess central stress and dopamine systems. However, other methods, such as microdialysis, are able to measure levels of neurochemicals under both basal and stress conditions within the same animal. Therefore, such methods should be utilized in future studies to better elucidate HPA-dopamine alterations in rats exposed to alcohol prenatally.

4.9 Translational potential of current findings

Although 100% preventable, FASD continues to be a significant societal problem. One preventative technique would be to make information about the effects of PAE more accessible to women of childbearing age. For example, spreading the message that the human embryonic

developmental stage frequently occurs prior to the recognition of pregnancy, and this stage is highly susceptible to the effects of PAE may prove to be highly valuable and preventative information to women of childbearing age. The findings from the current dissertation demonstrate life-long alterations in highly important brain systems (stress and dopamine) following PAE, and sharing the message that FASD is a developmental disorder with life- long effects may add to women's understanding of the implications of high alcohol consumption during periods when pregnancy may occur (Tough et al., 2005). The Canada FASD Research network is one example of an organization that promotes the message of 'practicing pregnancy' as it pertains to healthy life style including avoidance of binge use of substances as a FASD prevention technique, and the current dissertation advocates this message.

An immediate application, and perhaps one of the most important, is through the sharing of research findings to individuals with an FASD, their family members and other professionals, which provides scientific validation that FASD effects on the brain are widespread. This knowledge enhances understanding that these individuals are not choosing to 'act poorly', but rather are responding to their best of abilities while dealing with significant neurobiological alterations. Understanding HPA-dopamine interactions informs understanding of vulnerability to substance use disorders, and the present results demonstrate that these interactions occur differently in a brain exposed to alcohol *in utero,* which often parallel former studies demonstrating similar alterations following early life adversity. Health care professionals could greatly benefit from the knowledge that HPA-dopamine interactions are altered by PAE, and understanding these alterations could increase insight into specialized interventions and treatments for substance use disorders. Specifically, past and current results highlight the importance of early life interventions for preventing substance use disorders, such as

reducing/eliminating unnecessary stress and increasing stability in the lives of individuals with an FASD, especially during adolescence and early adulthood. Individuals with a FASD and substance use disorder would also likely benefit from specialized stress reduction techniques specific to her/his life. Additionally, coping strategies to better deal with stress, or increase the perception of control and/or predictability over stress, would be especially beneficial for treatment of substance use disorders for individuals with an FASD. Providing individuals who are going through treatment for substance use disorders with information about HPA-dopamine interactions may have therapeutic potential, and would assist with self-understanding and benefit treatment success.

4.10 Future Directions

A highly beneficial future direction would be to investigate the synaptic location and type of neuron on which the dopamine receptors are located following either chronic variable stress or repeated amphetamine exposure. For example, one potential mechanism through which problematic behaviour may arise is through GABAergic function (inhibitory). Glutamatergic outputs (excitatory) can be influenced by several sources of incoming information via GABAergic modification, allowing for enhanced behavioural plasticity (Seamans and Yang, 2004). In rodent studies, an increase in dopamine receptor expression (D_1) on glutamatergic projections to the nucleus accumbens is observed following repeated drug exposure (Kalivas et al., 2005), resulting in biased decision making for increased drug-seeking behaviour (reviewed in (Andersen and Teicher, 2009). The present dissertation did not observe baseline alterations in

dopamine receptor expression, but there was a drug-induced increase in D_1 and D_2 expression within the medial prefrontal cortex of PAE males but not PAE females. However, the type of neuron (e.g. GABA-ergic interneurons) as well as synaptic location (e.g. synaptic- or extra-synaptic) on which of dopamine receptors were located was not assessed and clearly plays a significant role in biasing neurocircuitries and the etiology of substance use disorders. Thus, it cannot be ruled out that either chronic variable stress, repeated amphetamine exposure, or repeated saline injection stress may enhance D_1 expression on glutamatergic projections to the nucleus accumbens in PAE subjects, which would theoretically bias decision-making towards addictive-like behaviours. Interestingly, men with a former substance use disorder exhibited significantly decreased dopaminergic receptors (D_2) compared to men without a history of substance use disorders (Volkow et al., 2009). In the present findings, PAE males, but not females, exhibited decreased dopamine receptors following repeated saline injections and stimulated dopamine release, suggesting that the relationship between decreased receptors and increased substance use disorders may be a sexually-dimorphic effect and warrants further investigation.

Although dopamine is strongly implicated in the substance use research, opioid, glutamatergic, GABAergic, and serotonergic systems are likely of equal importance in understanding the propensity towards addiction in models of FASD. For instance, increases in alcohol preference in prenatally alcohol-exposed rats was blocked by the co-administration of an opioid receptor antagonist, naltrexone (Arias and Chotro, 2005b). Nalaxone (similar to naltrexone) resulted in decreased opioid binding in the striatum (Shah and West, 1983). Additionally, glutamatergic function appears to be altered by PAE, as evidenced by altered expression and binding of receptors (Honse et al., 2003a, Honse et al., 2003b, Nixon et al.,

2002). These additional neurotransmitter systems should be studied further in the future in order to build a more comprehensive understanding of stress and dopamine system dysregulation following PAE.

Early life adversity and PAE: Adolescent chronic stress is an important area requiring further research. Given the often variable environments that young children with an FASD experience early in life, animal models should incorporate this into models. Modeling a 'double insult' would provide additional face validity to the common early life experiences of many children with FASD. For example, the timing of the chronic variable stress paradigm utilized could be applied at varying time points within adolescence and into early adulthood, in order to look at the effects of early life stress as well as timing of adversity on long-lasting alterations in adulthood. Additionally, the duration and intensity of the chronic variable stress paradigm could be manipulated in order to investigate the effects of varying lengths of repeated stress as well as severity on PAE effects.

Experience can have profound effects on the CNS, particularly early in life when neurobiological systems are still developing and/or maturing. Indeed, rather than the static process once believed, brain development is an ongoing process that is continuously modified and molded by experiential factors. Early life experiences may impact/influence brain development beginning early in gestation when the neural tube is developing and through until late adolescence/early adulthood when the brain finally reaches maturation. Importantly, each phase of early development sets the stage for the next phase. It follows then those environmental factors that influence brain development at one point will have the potential to shape subsequent developmental processes. In this manner, the final phenotype may be 'stamped' or imprinted by

early life events allowing the brain to be adapted to the environment through experience. For this reason, although brain plasticity, or the ability of the brain to be modified by experience, certainly extends well into later life, experience appears to have a much more profound and persistent influence on the developing brain.

However, a plastic brain is also a vulnerable brain. Adverse experiences are equally or perhaps even more, influential as favorable experiences in shaping the developing brain. Through the developmental process early life experience can shape the brain in such a way that either resilience or vulnerability to future mental health problems is increased. Environmental stimuli can activate the developing CNS in such a way that deficits in cognitive and behavioural performance can be either rescued or further perturbed. For example, in rats separation of pups from the dam for several minutes over a number of days (i.e. early handling) has been shown to positively influence the maturing HPA axis in such a way that enhances later life stress responsiveness (Weinberg & Levine, 1976; Meaney et al., 1985). As well, early handling ameliorates some, but not all, deficits related to HPA dysfunction in PAE offspring (Gallo & Weinberg, 1982; Weinberg et al., 1995; Gabriel & Weinberg, 2001; Gabriel et al., 2005). These studies suggest individuals with FASD may benefit from early environmental and behavioural interventions. The effects of PAE would likely be exacerbated by additional early life adversity, and stability and support during this vulnerable developmental period may serve as a protective factor for individuals with a FASD. Future studies are needed to identify highly plastic developmental periods for particular behavioural/cognitive problems associated with FASD, as well as potential interventions that are effective at reducing these problematic symptoms.

Interventions affecting whole systems: Although further investigation of multiple neural systems is beneficial, it is unlikely that alterations in a single neural system will rescue several facets of PAE's adverse effects. The current dissertation extends past research to further demonstrate that PAE produces significant long-lasting alterations in several neural systems. It is unlikely that a pharmacological intervention targeting a single neurotransmitter system will produce maximal benefits for problematic aspects of FASD. Interventions targeting multiple systems may be more effective at producing a wide spread shift. Based on past pre-clinical research, some highly promising avenues for interventions affecting multiple systems include: 1) reduction of maternal and early life stress (Schneider et al., 2002); 2) increase stability and overall quality of early life environment, as early environmental enrichment has been shown to reduce PAE effects on the brain (Rema and Ebner, 1999); 3) enhance maternal and child's nutritional intake, specifically pertaining to nutrients essential for epigenetic modification that are known to be reduced by PAE such as choline and folate (Lillycrop et al., 2005, Thomas et al., 2007); 4) specialized exercise programs to produce increased blood flow to the brain as well as widespread metabolic and motor effects known to be altered by PAE (Christie et al., 2005, Thomas et al., 2008); 5) specialized cognitive-behavioural therapies (CBT) as individuals with FASD and psychiatric illness tend to be more resistant to traditional CBTs (O'Connor et al., 2002). Co-use of pharmaceuticals alongside multiple system interventions may be needed to initiate or enhance the therapeutic intervention, such as the use of methylphenidate (Choong and Shen, 2004a), especially in more severe cases of FASD. Based on the present results of this dissertation as well as past evidence, pharmaceuticals should not be used as a sole treatment strategy for individuals with a FASD. Although pharmaceutical interventions may be more effective at producing relatively large shifts in targeted systems within a shorter time frame,

multiple system interventions may in fact be more effective at producing more widespread shifts in regulation within multiple neural circuitries.

4.11 Conclusions

The initial hypotheses of the present dissertation were that PAE: 1) would alter stress-induced changes in stress and dopamine systems; 2) would enhance sensitivity of underlying dopamine systems to repeated stimulant exposure; and 3) would enhance stimulant-induced changes in HPA function. Additionally, I expected to observe sex differences in the effects of PAE, stress, and drug exposure on stress signaling and dopamine receptor expression in key brain regions. Indeed, chronic variable stress produced more widespread alterations in stress signaling in PAE females and males, and attenuated the typical decrease in dopamine receptor expression following chronic stress exposure. Sex differences were observed in the regions and neural measures altered by chronic variable stress, with CRH mRNA in the medial prefrontal cortex and the posterior bed nucleus stria terminalis altered in females only and MR mRNA more affected in the hippocampus of males. Sensitivity of underlying dopamine systems to repeated amphetamine exposure was demonstrated in PAE females and males, and this corresponded with enhanced sensitivity of the HPA axis to acute stress. Interestingly, the effects of PAE on cross-sensitization between amphetamine and acute stress occurred differentially in males and females, with enhanced ACTH in PAE males but enhanced CORT in PAE females.

These results have implications for understanding a range of mental health problems that are known to be related to stress and dopamine systems, including substance use disorders,

depression, anxiety, schizophrenia, bipolar disorder, and several neurodegenerative disorders. Furthermore, these mental health problems occur at a higher incidence among individuals with FASD than the general public (O'Connor and Paley, 2009). Thus, it is vitally important to understand how PAE alters the underlying neurobiological mechanisms implicated in mental health problems in males and females in order to provide specialized prevention, intervention and treatment for this unique population.

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