PRENATAL ALCOHOL EXPOSURE AND CHRONIC STRESS IN ADULTHOOD: EFFECTS ON ANXIETY- AND DEPRESSIVE-LIKE BEHAVIOR AND CENTRAL REGULATION OF THE NEUROENDOCRINE STRESS RESPONSE

by

Vivian Yin Yin Lam

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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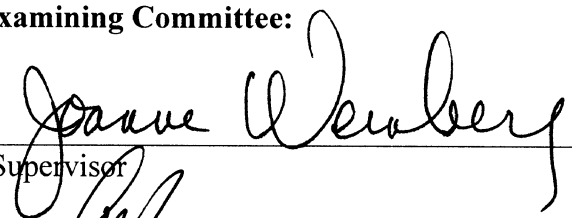


Dr. Joanne Weinberg

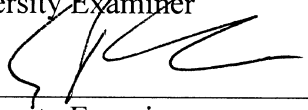
Supervisor

Dr. Liisa Galea

Supervisory Committee Member

Dr. Anita DeLongis

University Examiner



Dr. Stan Floresco

University Examiner

Abstract

Individuals prenatally exposed to alcohol show higher rates of mental health problems than unexposed individuals, with depression and anxiety being among the more commonly encountered disorders. Depression and anxiety are often observed in the context of stress and/or a dysregulated stress response system (the hypothalamic-pituitary-adrenal [HPA] axis). Prenatal alcohol exposure (PAE) can dysregulate the HPA axis, resulting in hyperresponsivity to stress, which in turn may predispose exposed individuals to the adverse effects of stress compared to unexposed individuals. With the overarching aim to examine the role of PAE-induced HPA hyperresponsivity to stress in depression and anxiety, and how sex differences may influence outcome, I examined the effects of PAE and chronic unpredictable stress (CUS) on multiple aspects of HPA regulation and behavioral output. I found that PAE alone altered, in a sex-dependent manner, baseline anxiety-/depressive-like behavior as well as neural activation and stress-related receptor expression in brain regions involved with both stress and emotional regulation compared to control animals. Additionally, PAE animals show differential sensitivity to the effects of chronic stress compared to unexposed animals. Furthermore, I demonstrate that several effects of CUS may have a delayed onset. Following the findings of these studies, I more directly examined the role of hypersecretion of corticosterone in mediating the effects of PAE and CUS on brain and behavior, by clamping corticosterone at basal physiological levels via adrenalectomy and hormone replacement in drinking water (ADXR). Our results suggest that while HPA hyperreactivity to stress is a robust consequence of PAE, corticosterone levels may be relevant for behavioral outcome and HPA regulation of PAE females but not males. Overall, our findings have important implications for understanding the role of stress and hormone

secretion in the adverse effects of PAE, which has clinical relevance as individuals prenatally exposed to alcohol are at a higher risk than unexposed individuals of encountering stressful environments during their lifetimes.

Lay Summary

Prenatal alcohol exposure (PAE) affects fetal brain development and causes a wide range of cognitive and behavioral deficits, which are described under the term Fetal Alcohol Spectrum Disorder. (FASD). In addition, affected individuals are more likely to show higher rates of depression and anxiety than unexposed individuals. However, the factors that contribute to this increased risk following PAE remain largely unknown. Depression and anxiety are often observed in the context of stress and/or an altered stress response system, and PAE can result in increased secretion of stress hormones in response to a stressor. This dissertation presents new evidence that susceptibility to stress later in life is higher following PAE, likely due to changes in how the stress response is regulated. Furthermore, the findings indicate that the hyper-response to stress induced by PAE may be relevant for outcome in females but not males.

Preface

Please note that all data chapters in this dissertation (Chapters 2-4) are presented in manuscript format, as they are currently published (Chapters 2 and 3), or in revision (Chapter 4).

A version of **Chapter 2** has been published as: Lam VYY, Raineki C, Takeuchi LE, Ellis L, Woodward TS, Weinberg J. Chronic Stress Alters Behavior in the Forced Swim Test and Underlying Neural Activity in Animals Exposed to Alcohol Prenatally: Sex- and Time-Dependent Effects. Front. Behav. Neurosci. 12:42. J. Weinberg, C. Raineki, L. Ellis and I designed the experiment. I executed all animal experiments, with the assistance of C. Raineki and L. Ellis. L. I collected the data, with the assistance of L. Takeuchi. I analyzed the data statistically, with the assistance of T. Woodward for the statistical technique constrained principal component analysis. I wrote the manuscript. C. Raineki and J. Weinberg provided critical feedback and all authors provided suggested edits prior to submission.

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A version of **Chapter 4** has been submitted and reviewed, and is currently under revision for resubmission as: Lam VYY, Raineki C, Ellis L, Yu W, Weinberg J. Role of corticosterone in anxiety-like behavior and HPA regulation following prenatal alcohol exposure. Progress in Neuro-psychopharmacology & Biological Psychiatry. I executed all animal experiments with the assistance of C. Raineki, L. Ellis, and W. Yu. I analyzed the data statistically and wrote the manuscript. C. Raineki and J. Weinberg provided critical feedback and all authors provided suggested edits prior to submission.

The animal studies presented in this thesis were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of British Columbia Animal Care Committee (certificates: A10-0116, A10-0136).

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
ADX	Adrenalectomy
ADXR	Adrenalectomy and Corticosterone Replacement
BDNF	Brain-derived Neurotrophic Factor
C	Control
Cg1	Anterior Cingulate
CPCA	Constrained Principal Component Analysis
CRHR1	Corticotropin-Releasing Hormone Receptor Type 1
CUS	Chronic Unpredictable Stress
CUS-1	Testing Initiated 1 Day Following Chronic Unpredictable Stress
CUS-14	Testing Initiated 14 Days Following Chronic Unpredictable Stress
DG	Dentate Gyrus
ERK	Extracellular Signal-Regulated Kinase
FASD	Fetal Alcohol Spectrum Disorder
GD	Gestational Day
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal
IL	Infralimbic Cortex
mpdPVN	Paraventricular Nucleus (Medial Parvocellular Dorsal Division)
mPFC	Medial Prefrontal Cortex

MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
NAc	Nucleus Accumbens
Non-CUS	Non-stressed
PAE	Prenatal Alcohol Exposure
PCA	Principal Component Analysis
PF	Pair-Fed
PND	Postnatal Day
PrL	Prelimbic Cortex
Trk B	Trypomyosin Receptor Kinase B

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Chapter 1: Introduction

1.1 General overview and hypotheses

Children and adults prenatally exposed to alcohol show higher rates of mental health problems than unexposed individuals, with depression and anxiety being among the more commonly encountered disorders (Famy et al., 1998; O'Connor and Paley, 2009; Pei et al., 2011). Preclinical studies support clinical findings and showed that prenatal alcohol exposure (PAE) can indeed increase depressive- and anxiety-like behavior in adulthood (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Varlinskaya and Mooney, 2014; Wilcoxon et al., 2005). However, depression and anxiety are often observed in the context of stress and/or a dysregulated stress response system (the hypothalamic-pituitary-adrenal [HPA] axis) (Jacobson, 2014; Nestler et al., 2002). Both clinical and preclinical studies demonstrated that PAE can result in HPA axis dysregulation, reporting increased HPA activation and/or a delayed return to basal levels, as well as altered central HPA regulation in PAE compared to control offspring (reviewed in (Hellemans et al., 2010a)). Therefore, I hypothesized that PAE-induced HPA hyperresponsivity to stress confers increased susceptibility to stress effects in adulthood on brain and behavior, and in turn, increases vulnerability to depression and anxiety. This has important clinical relevance as individuals prenatally exposed to alcohol are more likely than unexposed individuals to encounter stressful environments/experiences during their lifetimes (reviewed in (Hellemans et al., 2010a)).

By contrast to the general population (Angst and Dobler-Mikola, 1985; Cyranowski et al., 2000; Grant et al., 2005; Grigoriadis and Robinson, 2007; Hyde et al., 2008; Kessler, 2003; Kessler et

al., 2006, 2005; Kuehner, 2003; Piccinelli and Wilkinson, 2000; Regier et al., 1990; Spitzer et al., 2006; Weissman and Klerman, 1977; Weissman and Olfson, 1995; Wittchen et al., 1994), in which depression and anxiety occur more frequently in women than men, it is unclear whether sex differences in risk for depression and anxiety exist for individuals who were prenatally exposed to alcohol. One study on mental illness with a small sample size (15 men and 10 women who were prenatally exposed to alcohol) found that the rate of depression may be equal between men and women, while the rate of anxiety may be higher in women (Famy et al., 1998). Further studies examining the role of sex differences in outcome in the context of PAE would be clinically relevant and beneficial.

With the overarching aim to examine the role of PAE-induced HPA hyperresponsivity to stress in depression and anxiety, and how sex differences may influence outcome, I examined the effects of PAE and chronic unpredictable stress (CUS) on multiple aspects of HPA regulation and behavioral output. Specifically, I aimed to identify changes in activation of neural networks and expression of central stress-related receptors involved with both stress and emotional regulation that might be associated with sex-dependent alterations in depressive- and anxiety-like behavior following PAE and CUS. We previously found that CUS in adulthood increased anxiety- and depressive-like behavior in PAE rats compared to controls, with differential effects on male and female offspring (Hellemans et al., 2010a), and that PAE male and female rats activate different neural networks than control animals in response to the elevated plus maze, a stressor that has an emotional or anxiety-related component (Raineki et al., 2014). Building on these findings, I investigated the immediate and delayed effects of CUS on brain and behavior to gain insight into the changes that may occur immediately following chronic stress and those that

occur during a recovery period in the absence of repeated stress exposure, thus providing a more comprehensive and clinically relevant understanding of the effects of chronic stress in the context of PAE. I hypothesized that CUS would differentially increase depressive- and anxiety-like behavior in PAE compared to control animals and that differential neural networks and central expression of stress-related receptors in response to CUS could be identified. Given that others have found differential changes in brain and behavior between immediate and delayed testing following chronic stress or corticosterone exposure (Gourley and Taylor, 2009; Matuszewich et al., 2007) in control animals, I expect that some effects of CUS on PAE animals might appear only with delayed testing.

Following these studies and using the findings to help guide the selection of parameters to investigate, I more directly examined the role of HPA hyperresponsivity to stress – in particular, hypersecretion of corticosterone – in mediating the effects of PAE and CUS on brain and behavior, by clamping the corticosterone response to stress at basal physiological levels via adrenalectomy and hormone replacement in drinking water (ADXR), a procedure that can approximate the circadian rhythm of corticosterone. Here, I hypothesized that ADXR would attenuate or normalize differences between PAE and control animals, revealing a role for corticosterone in mediating the effects of PAE and CUS on anxiety-like behavior and glucocorticoid receptor expression. Taken together, elucidation of the neurobiological mechanisms by which PAE may, in a sex-dependent manner, increase vulnerability to the effects of stress and susceptibility to depression and anxiety, possibly through alterations in corticosterone secretion, may help identify and develop novel and sex-specific intervention targets.

1.2 Fetal alcohol spectrum disorder

Alcohol is a teratogen that affects fetal brain and body development (Jones and Smith, 1973; Lemoine et al., 2003; Mattson and Riley, 1998; Streissguth et al., 1991) and depending on the amount and timing of exposure *in utero*, alcohol causes a wide range of adverse effects in alcohol-exposed offspring. These effects are described under the umbrella term "fetal alcohol spectrum disorders" (FASD). At the most severe end of the continuum is fetal alcohol syndrome (FAS), which typically occurs following chronic consumption of high doses of alcohol. FAS is characterized by distinct craniofacial dysmorphology, pre- and postnatal growth retardation, neurological abnormalities, and cognitive deficits (Manning and Eugene Hoyme, 2007; Riley and McGee, 2005). Exposure to alcohol doses that does not produce the facial characteristics but results in some diagnostic features of FAS can lead to a diagnosis for partial FAS (pFAS), alcohol-related birth defects (ARBD), or alcohol-related neurodevelopmental disorder (ARND) (Hoyme et al., 2016). An alternative diagnostic term for ARND is "neurobehavioral disorder associated with prenatal alcohol exposure" (ND-PAE) and is based on 3 major areas of impairments following PAE regardless of presence of physical deficits: neurocognition, self-regulation, and adaptive functioning (Hagan et al., 2016). The estimated prevalence of FASD is 31 to 99 in 1000 children in the United States (May et al., 2018) and 7.7 in 1000 people globally (Lange et al., 2017). Across the spectrum, cognitive, neuropsychological, and behavioral problems (Astley et al., 2009b; Mattson et al., 2001; Shaywitz et al., 1980; Streissguth, 1986) are consistently observed.

In addition to the aforementioned effects, children and adults with a FASD frequently develop one or more mental health issues, with depression and anxiety being among the most commonly

encountered psychiatric problems (Fryer et al., 2007; O'Connor and Paley, 2009). One statistic suggests that in British Columbia, >90% of adults with a FASD have a mental health diagnosis, and depression makes up greater than 40% of these diagnoses (Clark et al., 2004), while about 20% meet criteria for an anxiety disorder (Famy et al., 1998). Previous studies in rats showed that prenatal alcohol exposure (PAE) can indeed increase depressive- and anxiety-like behavior (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Varlinskaya and Mooney, 2014; Wilcoxon et al., 2005). However, depression and anxiety are often observed in the context of stress and/or a dysregulated stress response system (the hypothalamic-pituitary-adrenal [HPA] axis) (Jacobson, 2014; Nestler et al., 2002). Programming of the HPA axis by adverse prenatal or early life events may mediate, at least in part, the relationship between the developmental environment and later life susceptibility to stress-related disorders (Hellemans et al., 2010a; Matthews, 2000; Meaney et al., 2007; Phillips et al., 2000, 1998).

1.3 Dysregulation of the hypothalamic-pituitary-adrenal axis in depression and anxiety

The HPA axis is a major neuroendocrine stress response system sensitive to physical and psychological stressors that perturb homeostasis as well as circadian signals from the suprachiasmatic nucleus (Herman et al., 2016, 2005, 2003; Moore and Eichler, 1972). HPA activity is often found to be sec dependent across species, with greater HPA responses and resistance to negative feedback regulation in females than males (Herman et al., 2016; Young, 1998).

Activation of the HPA axis begins with the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP, which potentiates the activity of CRH) from the medial parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus into the hypothalamic hypophysial portal system (Herman et al., 2016). CRH and AVP then trigger the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn, stimulates glucocorticoid synthesis and secretion from the adrenal cortex (Herman et al., 2016). Glucocorticoids (cortisol in human, corticosterone in rats) regulate availability and utilization of energy substrates and provide negative feedback to the hypothalamus and pituitary to shut down the stress response, as well as act on central brain regions that regulate HPA axis activity such as the prefrontal cortex, amygdala, and the hippocampal formation (Herman et al., 2016).

Three receptors in the brain are key in regulating HPA activity: the corticotropin-releasing hormone receptor type 1 (CRHR1), mineralocorticoid receptor (MR), and glucocorticoid receptor (GR). CRHR1 is one of the receptors that mediates the neuroregulatory effects of CRH, whereas MR and GR mediate the effects of glucocorticoids. CRHR1, activated by CRH, can both facilitate and depress neurotransmission (Gallagher et al., 2008). This receptor is widely expressed in the brain, including limbic regions such as the medial prefrontal cortex (mPFC), amygdala, and hippocampal formation (Henckens et al., 2016), and it has different roles in modulating HPA activity and behavior depending on where it is expressed. MR expression is confined to limbic areas, mostly in the hippocampus, whereas GR is more widely expressed in the brain (Ahima et al., 1991; Ahima and Harlan, 1990; Aronsson et al., 1988; Arriza et al., 1988; Fuxe et al., 1985; Holsboer, 2000; Matthews et al., 2002; Reul and de Kloet, 1986). Cytoplasmic MR has a high affinity for glucocorticoids and is responsive to basal levels (Reul

and de Kloet, 1985). However, membrane-bound MR has been found to have a lower affinity for glucocorticoids than cytosolic MR and as such may be available to respond to high levels of glucocorticoids during stress (ter Heegde et al., 2015). MR is involved in regulating basal HPA tone and in setting the threshold of HPA reactivity to stress and maintaining high neuronal excitability (Joëls and de Kloet, 2017; ter Heegde et al., 2015). Furthermore, transgenic and pharmacological studies indicate that MR may be crucial for promoting neurogenesis, maintaining neuronal integrity, and preventing GR-mediated apoptosis (ter Heegde et al., 2015). In contrast, the lower-affinity GR is only activated during intermediate to high glucocorticoid levels (e.g. during the glucocorticoid circadian peak or in response to stress) (Reul and de Kloet, 1985), and primarily mediate feedforward/feedback regulation of the stress response (Herman et al., 2016).

Short-term activation of the HPA axis is adaptive; however, persistent elevation of basal HPA tone due to frequent or sustained activation or an inability to shut off the stress response in a timely manner has maladaptive consequences on health and well-being (McEwen, 2004). HPA tone is increased in a significant proportion of individuals with depression: salivary, plasma, and urinary free cortisol levels, as well as mean 24-hr plasma ACTH levels are increased (Deuschle et al., 1997; Dinan and Scott, 2005; Gold and Chrousos, 2002; Holsboer, 2001, 2000; Nemeroff and Vale, 2005; Nestler et al., 2002; Parker et al., 2003; Swaab et al., 2005). Morning awakening cortisol level is higher in individuals with major depression than those without (Dietrich et al., 2013; Ulrike et al., 2013), and higher levels are associated with increased risk for depression (Adam et al., 2010). The cortisol diurnal variation may also be smaller in depression: morning

awakening level is slightly elevated while the circadian nadir level is almost two times higher in depressed patients than healthy controls (Deuschle et al., 1997; Wong et al., 2000).

Deficits in GR-mediated feedback inhibition, impaired MR function, and increased sensitivity to CRH may contribute to HPA dysregulation. Whereas administration of dexamethasone, a synthetic glucocorticoid that binds GR, suppresses cortisol secretion in healthy controls, it is ineffective in patients with depression (Carroll, 1984; Vreeburg et al., 2009) or anxiety (Brawman-Mintzer and Lydiard, 1997). Cortisol and ACTH suppression can be achieved though at high dexamethasone doses in individuals with depression (Modell et al., 1997), providing support that sensitivity to glucocorticoids in GR-mediated negative feedback may be decreased in depressed patients in comparison to controls (Holsboer, 2000). Those with depression are also less responsive to feedback inhibition by the MR agonist fludrocortisone (Lembke et al., 2013). Antagonism with spironolactone on the other hand revealed higher cortisol levels in depressed patients than healthy controls (Young et al., 2003). Furthermore, while spironolactone is effective in increasing cortisol levels compared to placebo in controls, it has no effects in patients with depression (Juruena et al., 2013). In the dexamethasone/CRH test, which is more sensitive than the dexamethasone test (Deuschle et al., 1998; Heuser et al., 1994; Watson et al., 2006), plasma levels of cortisol and ACTH in response to CRH infusion following dexamethasone administration are higher in depressed patients than control subjects (Nemeroff, 1996; Pariante and Miller, 2001).

In addition to altered activity, GR and MR expression are reduced in post-mortem brains of depressive patients (Klok et al., 2011; López et al., 1998; Medina et al., 2013; Webster and

Carlstedt-Duke, 2002). Moreover, antidepressant treatment has been found in both animal and *in vitro* studies to upregulate GR and MR expression and function, as well as decrease basal and stress HPA activity (Pariante, 2004; Pariante and Miller, 2001). Treatment with GR or MR agonists (Bouwer et al., 2000; DeBattista et al., 2000; Dinan et al., 1997) or GR antagonists (Belanoff et al., 2001) also have antidepressant effects.

Thus far, the discussion has been focused primarily on the neurobiological underpinnings of depression. However, etiology and symptomatology of anxiety may potentially share common neurobiological pathways with depression, as anxiety may be treated with antidepressants (Kalueff and Nutt, 2007; Nutt et al., 2002). Also, as with depression, dexamethasone is less effective in individuals with anxiety (Schweizer et al., 1986; Tiller et al., 1988), cortisol levels are higher in affected individuals than healthy controls (Mantella et al., 2008; Pomara et al., 2005; Tafet et al., 2005), and individuals with persistent anxiety have higher morning cortisol and a higher cortisol awakening response (Greaves-Lord et al., 2007). Furthermore, it is important to note that depression and anxiety are highly comorbid (Hellemans et al., 2010a; Masi et al., 2004), and morning plasma and salivary cortisol levels are also elevated in individuals with anxiety and comorbid depression, with higher levels associated with an increased severity of anxiety and depression (Funke et al., 2017; Nelemans et al., 2013). Additionally, individuals with co-morbid depression and anxiety may hypersecrete ACTH in response to a social stressor (Young, 2004).

1.4 HPA dysregulation following prenatal alcohol exposure

1.4.1 PAE causes increased HPA drive and deficits in feedback inhibition

The fetal HPA axis is highly susceptible to programming by PAE, resulting in altered HPA activity and regulation that parallel many of the changes observed in depression. Higher basal and stress cortisol levels have been reported in infants (Haley et al., 2006; Jacobson et al., 1999; Ramsay et al., 1996), and studies using animals have demonstrated increased HPA activation and/or a delayed return to basal levels as well as altered central HPA regulation in PAE compared to control offspring in response to a wide range of stressors (Lee et al., 2000, 1990; Lee and Rivier, 1996; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1988; Weinberg et al., 2008). HPA tone is increased in infants prenatally exposed to alcohol, manifest as heightened basal and post-stress cortisol concentrations and elevated heart rate compared to control infants (Haley et al., 2006; Jacobson et al., 1999; Ramsay et al., 1996). Although children and adolescents with FASD exhibited similarly high awakening cortisol levels, their cortisol levels during the circadian trough are higher than typically developing controls, suggesting decreased cortisol diurnal variation that is reminiscent of the cortisol circadian profile in depression (Keiver et al., 2015; McLachlan et al., 2016). Although rats prenatally exposed to alcohol typically show comparable corticosterone and ACTH levels as unexposed controls under basal conditions, levels of these hormones are found to be higher in response to stress (Hellemans et al., 2010a).

Increased HPA drive and deficits in feedback inhibition may account for HPA dysregulation following PAE. Increased HPA drive in PAE animals is reflected by increased steady state hypothalamic CRH mRNA levels (Gabriel et al., 2005; Lee et al., 1990; Lee and Rivier, 1996;

Redei et al., 1989) and increased steady state anterior pituitary proopiomelanocortin (POMC) mRNA (POMC is the precursor of ACTH) (Redei et al., 1993). Higher basal ACTH levels (only in PAE males), greater hypothalamic CRH mRNA levels and lower pituitary CRH-R1 mRNA levels are unmasked in PAE animals following an adrenalectomy (ADX) challenge (Glavas et al., 2007). Unlike what is observed in depression, ACTH levels are not different between control and PAE animals in response to the CRH challenge test without dexamethasone pretreatment (Lee et al., 2000).

Deficits in glucocorticoid feedback regulation following PAE is demonstrated by increased HPA response to dexamethasone blockade (Osborn et al., 1996) and the dexamethasone/CRH test (Osborn et al., 2000). MR activity may also be higher in PAE than control subjects, as MR blockade resulted in significantly greater ACTH release in PAE females compared to control counterparts (Glavas et al., 2006). Furthermore, MR sensitivity to corticosterone feedback regulation may be decreased, as corticosterone replacement following ADX does not normalize ADX-induced increase in hippocampal MR mRNA in PAE males (Glavas et al., 2007). Taken together, the parallel HPA dysregulation observed in both depression and FASD suggests a possible role for HPA axis dysfunction in mediating the increased vulnerability to develop depression and anxiety in individuals with a FASD.

1.4.2 Sex differences in the effects of PAE on the HPA axis

As noted, studies in both human infants and animal models have shown that PAE reprograms the fetal HPA axis, such that basal HPA tone is increased, and offspring are hyperresponsive to stressors (Haley et al., 2006; Jacobson et al., 1999; Ramsay et al., 1996). Importantly, these

effects were sex dependent, with girls showing greater changes in heart rate and negative affect, and boys showing greater changes in cortisol. Sex differences are often observed in animal models as well, depending on the nature and intensity of the stressor, time course of testing, and hormonal or behavioral endpoint (Hellemans et al., 2010a; Weinberg et al., 2008). For example, the combination of moderate prenatal alcohol and noise stress reduced birth weights in male but not female rhesus monkey offspring, although both males and females showed increased HPA responses to maternal separation (Schneider et al., 2005). Our studies have also shown that sex differences in HPA responses of male and female offspring might be mediated by alcohol-induced changes in sex hormone activity and/or altered interactions between HPA and sex hormones. In males, PAE altered both HPA and sex hormone regulation, and reduced sensitivity of the HPA to the inhibitory effects of testosterone (Lan et al., 2009a). By contrast, HPA activity of PAE females was affected by the estrous cycle, with higher basal corticosterone levels during proestrus, the cycle phase when estradiol levels are high, than control females, and their response to stress was dependent on the phase of the cycle, suggesting differential sensitivity to the sex hormones in PAE compared to control females (Lan et al., 2009b; Sliwowska et al., 2008).

1.4.3 PAE effects on brain regions involved in regulating both HPA function and emotion

The prefrontal cortex and the hippocampus have been implicated in inhibiting HPA axis activity and terminating the stress response, whereas the infralimbic cortex of the prefrontal cortex and the amygdala are involved in activating the HPA axis (Herman et al., 2005). These areas also play major roles in emotional regulation. The prefrontal cortex is involved with cognition and executive functioning (Salzman and Fusi, 2010), and the hippocampus is implicated in

declarative memory and spatial learning (Nestler et al., 2002); therefore, dysfunctions in the prefrontal cortex and hippocampus may contribute to the cognitive aspects of depression, such as memory impairments, and feelings of worthlessness, hopelessness, doom, guilt, and suicidality (Nestler et al., 2002). The amygdala is associated with the conditioned fear response, emotional learning and memory, and processing emotional valence and intensity (Nestler et al., 2002; Salzman and Fusi, 2010). Abnormalities in amygdalar function may mediate the loss of positive affect in depression and contribute to the symptoms of anhedonia (loss of interest/decrease drive for pleasurable activities), anxiety, and reduced motivation (Nestler et al., 2002).

There is evidence for structural or functional alterations in the prefrontal cortex, hippocampus, and amygdala in depression (Davidson et al., 2002). In the dorsal anterolateral and dorsomedial PFC of depressives, cerebral blood flow and glucose metabolism are lower than normal (Drevets, 1999). Hippocampal volume is reduced in some studies, with the degree of reduction related to the duration of illness (Bremner et al., 2000; Lloyd et al., 2004; Sheline et al., 1999, 1996). In the amygdala, resting cerebral blood flow and glucose metabolism are comparatively higher in depressed patients than in control subjects (Abercrombie et al., 1996; Drevets, 2000a; Drevets et al., 1992). When shown emotional stimuli, the amygdala of depressed patients is also more activated than controls (Fu et al., 2004; Siegle et al., 2002; Surguladze et al., 2005). Alterations in structure and function in the amygdala may relate to depression severity (Abercrombie et al., 1998). The changes in these regions in depressed patients are often reversible with effective antidepressant treatments (Davidson et al., 2002; Drevets, 2000b; Jacobs et al., 2000; Nestler et al., 2002).

PAE is known to damage the brain. Of relevance, the prefrontal cortex, hippocampus, and amygdala are structurally and functionally altered in FASD. Magnetic resonance imaging (MRI), MR spectroscopy, and functional MRI studies found functional abnormalities in the PFC of children with FASD (Astley et al., 2009b). MRI scans of children and adolescents with FASD also found that deep gray matter volume in the amygdala is reduced (Nardelli et al., 2011). Moreover, smaller hippocampi, before or after accounting for total brain volume (Archibald et al., 2001; Astley et al., 2009a; Coles et al., 2011; Lebel et al., 2011; Nardelli et al., 2011; Riikonen et al., 2005; Willoughby et al., 2008), and agenesis of the hippocampal commissure (Johnson et al., 1996; Swayze et al., 1997) are demonstrated in subjects with FASD. One study also showed that age-related increase in hippocampal volume is not observed in FASD subjects (Willoughby et al., 2008). Given these regions' role in depression and in HPA regulation, the increased vulnerability to develop depression in individuals with a FASD may be attributed to alterations in structure and function of these regions following gestational exposure to alcohol.

1.5 PAE and chronic stress effects on brain and behavior

The emergence of internalizing psychopathology in individuals prenatally exposed to alcohol has been suggested to be a result of exposure of individuals with FASD, who may be constitutionally vulnerable, to stressors in the postnatal environment (Carmichael Olson et al., 2001; Kovacs and Devlin, 1998; O'Connor and Kasari, 2000). This is of particular relevance as alcohol-exposed individuals are more likely than unexposed individuals to encounter stressful life events (O'Connor and Paley, 2006; Streissguth et al., 2004, 1991). Therefore, HPA dysregulation induced by PAE may predispose these individuals to an increased vulnerability to stress-related disorders such as anxiety or depression following stress over the life course.

In support, we have previously found using a rat model that PAE differentially affects males and females in tasks that are suggested to measure depressive-like behaviors – insensitivity to a change in reward value of sucrose (in males), increased immobility in the forced swim test (in females) and altered social interaction (in both males and females) – following CUS in adulthood (Hellemans et al., 2010a, 2010b). We have also shown that CUS effects on PAE animals are associated with changes in neural activation and/or mRNA expression of stress neuropeptides in limbic-forebrain regions that are involved in HPA regulation (Herman et al., 2005; Myers et al., 2012) – the medial prefrontal cortex (mPFC), amygdala, hippocampus and hypothalamus (Lan et al., 2015; Raineki et al., 2014). Specifically, in the paraventricular nucleus (PVN) of the hypothalamus, CUS increased corticotropin-releasing hormone (CRH) and arginine vasopressin mRNA expression in PAE compared to C males (Lan et al., 2015), and PAE males failed to show CUS-induced reduction in neural activation in the amygdala and hippocampal formation (Raineki et al., 2014). CUS also decreased basal CRH mRNA expression and neural activation in the mPFC of PAE, but not C, females. Using a neural network framework to investigate the effects of PAE on the neural activity of these areas, we have found that PAE male and female rats activate different neural networks than control animals in response to the elevated plus maze, a stressor that has an emotional or anxiety-related component (Raineki et al., 2014). Moreover, CUS in adulthood differentially affected neural networks in PAE compared to control males and females, suggesting sex-dependent effects of PAE and CUS (Raineki et al., 2014).

In humans, challenges exist in accurately determining the date of onset of psychopathological symptoms (Andrews, 1981), potentially exacerbated by a lag between first onset of symptoms and initiation of help-seeking. A median lag of 3 to 30 years for anxiety disorders and 1 to 14

years for mood disorders between onset of symptoms and treatment has been reported (Wang et al., 2007). The causal relationship between stressors and symptoms of mental health issues has also been suggested to potentially be "underestimated and downplayed" due to inadequacy in determining stress exposure in individuals (Ensel and Lin, 1996). Clinical studies have shown that the effects of a stressful event are strongest when the event occurred recently (Andrews, 1981; Brown and Harris, 1978; Ensel and Lin, 1996; Mclean and Link, 1994; Tennant et al., 1981). However, "distal" stressors/events that occurred as much as 15 years prior to diagnosis were found to contribute, to a lesser extent than recent events, to current levels of distress (Ensel and Lin, 1996). Extensive studies in rats and mice support clinical findings by showing that chronic exposure to mild stressors results in the immediate manifestation of several behaviors related to a depressive phenotype (Willner, 2005) as well as changes in several neurochemical systems (Hill et al., 2012). In addition, animal studies have also shown differential changes in behavior and brain depending on whether there was a recovery period between chronic stress or corticosterone exposure and testing (Gourley and Taylor, 2009; Matuszewich et al., 2007). These studies reported delayed changes in behavior found at two weeks or more following termination of stress exposure, along with concomitant alterations in several neurotrophin-related signalling and heat shock proteins involved in depression (Gourley et al., 2008b, 2008a; Matuszewich et al., 2007). Thus, brain and behavioral consequences of repeated or chronic stress may be temporally dynamic, with some changes that may occur immediately following chronic stress, while HPA activity was likely increased and basal levels of corticosterone were expected to be elevated (based on the reported effects of chronic stress on corticosterone levels in the literature (Goshen et al., 2008; Magariños and McEwen, 1995; Pitman et al., 1988)), and other changes that occur during a recovery period in the absence of repeated stress exposure and with potential

recovery of HPA activity. In the context of PAE, however, less is known about the immediate and persistent/delayed effects of stress. Investigation of both the immediate and the persistent/delayed impacts of chronic stress is highly relevant for a more thorough understanding of the etiology of stress-related disorders, such as depression and anxiety, following PAE.

1.6 Thesis overview

The overarching goal of this dissertation was to test the hypothesis that PAE-induced HPA hyperresponsivity to stress confers increased susceptibility to stress effects over the life course on brain and behavior. The experimental data will be presented in three chapters, addressing the specific aims outlined in section 1.1. **Chapter 2**, entitled "Chronic stress alters behavior in the forced swim test and underlying neural activity in animals exposed to alcohol prenatally: Sex- and time-dependent effects" aimed to characterize alterations in the neural activation profile of brain areas involved in regulating emotion and stress – the prefrontal cortex, amygdala, and hippocampal formation – that may underlie the interactive effects of PAE and CUS in adulthood on depressive-like behavior. **Chapter 3**, entitled "Interactive effects of prenatal alcohol exposure and chronic stress in adulthood on anxiety-like behavior and central stress-related receptor mRNA expression: Sex- and time-dependent effects" probed for changes in expression of central stress-related receptors, in the same brain areas as Chapter 2, that may be associated with changes in anxiety-like behavior following PAE and CUS. The same cohort of animals was used in Chapters 2 and 3. To build on and extend the findings of Chapters 2 and 3 to more directly investigate the role of the HPA – in particular, hypersecretion of corticosterone – in mediating the effects of PAE and CUS, we clamped the corticosterone response to stress at basal physiological levels via adrenalectomy and hormone replacement in drinking water, a procedure

that can approximate the circadian rhythm, and the findings are reported in **Chapter 4**, entitled "Differential role of corticosterone in anxiety-like behavior and central HPA regulation in animals prenatally exposed to alcohol compared to controls". Finally, in **Chapter 5**, the main findings from each data chapter will be integrated alongside a discussion of limitations and future directions for these studies.

Chapter 2: Chronic stress alters behavior in the forced swim test and underlying neural activity in animals exposed to alcohol prenatally: Sex- and time-dependent effects

2.1 Introduction

Data from both clinical and animal studies suggest that prenatal alcohol exposure (PAE) induces a wide range of adverse neural, cognitive, behavioral, and endocrine effects (Hellemans et al., 2010a; Mooney and Varlinskaya, 2011; Probyn et al., 2013; Riley et al., 2011; Schneider et al., 2011; Valenzuela et al., 2012). Clinically, individuals prenatally exposed to alcohol also often experience mental health problems; in particular, depression and anxiety are disproportionately higher in these individuals than in unexposed individuals (Famy et al., 1998; O'Connor et al., 2002; O'Connor and Paley, 2009; Pei et al., 2011; Streissguth et al., 2004, 1991). Dysregulation of the stress response system, the hypothalamic-pituitary-adrenal (HPA) axis, has been suggested to play a role in vulnerability to stress-related disorders, such as depression and anxiety (Jacobson, 2014; Nestler et al., 2002). As well, programming of the HPA axis by adverse prenatal or early life events may mediate, at least in part, the relationship between the developmental environment and later life susceptibility to stress-related disorders (Hellemans et al., 2010a; Matthews, 2000; Meaney et al., 2007; Phillips et al., 2000, 1998). The fetal HPA axis is highly susceptible to programming by PAE, resulting in altered HPA activity and regulation. Indeed, higher basal and stress cortisol levels have been reported in infants (Haley et al., 2006; Jacobson et al., 1999; Ramsay et al., 1996), and studies using animals have demonstrated increased HPA activation and/or a delayed return to basal levels as well as altered central HPA regulation in PAE compared to control offspring in response to a wide range of stressors (Lee et al., 2000, 1990; Lee and Rivier, 1996; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982;

Weinberg, 1988; Weinberg et al., 2008). Moreover, individuals prenatally exposed to alcohol are more likely than unexposed individuals to encounter stressful life events (O'Connor and Paley, 2006; Streissguth et al., 2004, 1991). Whether these individuals show higher susceptibility to the consequences of these stressful events is not fully understood. However, it is possible that PAE-induced HPA dysregulation may predispose these individuals to an increased vulnerability to stress-related disorders following subsequent exposure to stressors over the life course.

Using a rat model of PAE, we have previously found that PAE differentially affects males and females in tasks that are suggested to measure depressive-like behaviors – insensitivity to a change in reward value of sucrose (in males), increased immobility in the forced swim test (in females) and altered social interaction (in both males and females) – following chronic unpredictable stress (CUS) in adulthood (Hellemans et al., 2010a, 2010b). We have also shown that these effects of CUS are associated with changes in neural activation and/or mRNA expression of stress neuropeptides in limbic-forebrain regions that are involved in HPA regulation (Herman et al., 2005; Myers et al., 2012) – the medial prefrontal cortex (mPFC), amygdala, hippocampus and hypothalamus (Lan et al., 2015; Raineki et al., 2014). Specifically, in the paraventricular nucleus (PVN) of the hypothalamus, CUS increased corticotropin-releasing hormone (CRH) and arginine vasopressin mRNA expression in PAE compared to C males (Lan et al., 2015), and PAE males failed to show CUS-induced reduction in neural activation in the amygdala and hippocampal formation (Raineki et al., 2014). CUS also decreased basal CRH mRNA expression and neural activation in the mPFC of PAE, but not C, females (Raineki et al., 2014). Additionally, structural and functional abnormalities of these brain areas in humans have been associated with several mental health disorders, including

depression (Drevets et al., 2008; Krishnan and Nestler, 2010). Using a neural network framework to investigate the effects of PAE on the neural activity of these areas, we have found that PAE male and female rats activate different neural networks than control animals in response to the elevated plus maze, a stressor that has an emotional or anxiety-related component (Raineki et al., 2014). Moreover, CUS in adulthood differentially affected neural networks in PAE compared to control males and females, suggesting sex-dependent effects of PAE and CUS (Raineki et al., 2014).

In humans, it can be challenging to both accurately determine the date of onset of psychopathological symptoms (Andrews, 1981), and to measure stress exposure in individuals (Ensel and Lin, 1996). Clinical studies have shown that the effects of a stressful event are strongest when the event occurred recently (Andrews, 1981; Brown and Harris, 1978; Ensel and Lin, 1996; Mclean and Link, 1994; Tennant et al., 1981). However, "distal" stressors/events that occurred as much as 15 years prior to diagnosis were found to contribute, to a lesser extent than recent events, to current levels of distress (Ensel and Lin, 1996). Consistent with clinical findings, extensive studies in rats and mice have shown that chronic exposure to mild stressors results in the immediate manifestation of several behaviors related to a depressive phenotype (Willner, 2005) as well as changes in several neurochemical systems (Hill et al., 2012). Moreover, animal studies have also reported delayed effects following chronic exposure to mild stressors or corticosterone, with changes in behavior found at two weeks or more following termination of stress exposure, along with concomitant alterations in several neurotrophin-related signalling and heat shock proteins involved in depression (Gourley et al., 2008b, 2008a; Matuszewich et al., 2007). Thus, brain and behavioral consequences of repeated or chronic stress

may be temporally dynamic, but in the context of PAE, less is known about the immediate and persistent/delayed effects of stress. Investigation of both the immediate and the persistent/delayed impacts of chronic stress is highly relevant for a more thorough understanding of the etiology of stress-related disorders, such as depression, following PAE.

In the context of PAE, the present study aimed to examine systematically immediate and persistent/delayed effects of adult CUS on behavior of male and female rats in the forced swim test – a test often utilized to measure depressive-like or passive coping behavior – and the neurocircuitry underlying stress and emotional regulation. To determine possible underlying neurobiological changes that may subserve PAE and/or CUS effects, *c-fos* mRNA expression, as a measure of neural activation, was assessed in the mPFC, amygdala, hypothalamus, and hippocampal formation. Because of the multiple brain and behavioral measures, in addition to traditional univariate analyses, further analyses were carried out using Constrained Principal Component Analysis (CPCA), a multivariate exploratory and data reduction technique that allowed us to identify potential neural activation networks associated with interactive effects of PAE and CUS on behavior.

2.2 Materials and methods

2.2.1 Animals and breeding

All animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Canadian Council on Animal Care, and were approved by the University of British Columbia Animal Care Committee. Adult virgin male (275-300g) and female (265-300g) Sprague-Dawley rats were obtained from Charles

River Laboratories (St. Constant, PQ, Canada). The animal facility was maintained on a 12:12 hr light/dark cycle (lights on at 07:00 h), under controlled temperature (21±1°C). Rats were pair-housed by sex and given *ad libitum* access to standard laboratory chow and water while they habituated to the facility for a 7-10 days period. For breeding, males were singly housed, and a female and male were paired. Presence of sperm in vaginal lavage samples taken every morning at 08:00 hr indicated day 1 of gestation (GD 1).

2.2.2 Diets and feeding

On GD 1, females were singly housed in a new colony room in polycarbonate cages on ventilated racks with beta chip bedding. Dams were randomly assigned to one of three treatment groups: 1) Alcohol-fed (PAE; n=13), receiving a liquid ethanol diet with 36% ethanol-derived calories, 6.7% v/v; 2) Pair-fed (PF; n=11), receiving a liquid control diet with maltose-dextrin isocalorically substituted for ethanol, in an amount consumed by a PAE partner (g/kg body weight/day of gestation); and 3) *Ad libitum*-fed control (C; n=10), receiving a pelleted control diet. All diet formulations provided optimal nutrition during pregnancy (Weinberg/Keiver High Protein Ethanol [#710324] and Control [#710109] liquid diets, and Weinberg/Keiver High Protein Pelleted Control Diet [#710109] were prepared by Dyets, Inc., Bethlehem, PA, USA) (Lan et al., 2006).

The diets were presented fresh daily, 1 hr prior to lights off to minimize shifts in the maternal corticosterone circadian rhythm (Gallo and Weinberg, 1981), and at that time the volume of liquid diet consumed since the previous night was recorded. All groups also received *ad libitum* access to water. Liquid ethanol diets were introduced gradually over the first 2 days of pregnancy

with a 1:2 ratio of liquid ethanol to liquid control diet on GD 1 and a 2:1 ratio on GD 2 to facilitate the transition into a full liquid ethanol diet beginning on GD 3. On GD 17, blood samples were collected from the tail vein 3 hr after lights off from a subset of PAE, PF, and C dams (n=3 each) and blood alcohol levels were determined using an assay from Pointe Scientific Inc. (Canton, MI, USA). Experimental diets continued through GD 21. Beginning on GD 22 and throughout lactation, dams received *ad libitum* access to 19% protein laboratory chow (Teklad Global #2019) and water. At birth (postnatal day, PND 1), pups were weighed, and litters randomly culled to 12 (6 males, 6 females when possible). If necessary to maintain litter size, pups born on the same day, from the same prenatal treatment group, were fostered into a litter. Dams and offspring were weighed weekly but were otherwise undisturbed until weaning on PND 22, after which pups were group housed by litter and sex until they were pair-housed at ~PND 40. Animals of the same sex and from the same prenatal treatment, but from different litters born +/- 2 days apart were paired. Weaned pups and adults were fed an 18% protein chow (Teklad Global #2018) and housed on standard non-ventilated racks.

A cohort of offspring that received the same prenatal treatment was left undisturbed, except for weekly cage changing, until termination.

2.2.3 Chronic unpredictable stress (CUS) paradigm

Animals from each experimental group were randomly assigned to either stress (CUS) or no-stress (non-CUS) conditions. Only one male and one female offspring from each litter were assigned to any one condition. CUS involved 10 days (**FIGURE 2.1**) of twice daily unpredictable exposure to mild stressors at variable time and order, with a minimum of 2 hr

between stressors; by the end of the 10-day period, all CUS rats experienced each stressor the same number of times. This is a modified CUS protocol, shorter in duration than that described in the literature (Hill et al., 2012; Willner et al., 1987) and designed to produce primarily psychological stress. This is based on findings that robust and reliable effects of CUS have been suggested to present as early as 10 days following the onset of stress exposure (Hill et al., 2012), and that the HPA axis of PAE animals tend to be hyperresponsive to stressors (Hellemans et al., 2010a); therefore, a shorter 10-day model was employed to avoid potential ceiling effects in PAE subjects. Stressors used included: Platform: Animals individually placed on 20 x 20 cm transparent Plexiglas platforms elevated at a height of 90 cm. Restraint: Animals individually restrained in PVC tubes (15 cm × 6 cm for females and 19 cm × 7 cm for males) with ventilation holes for 30 min. Soiled Cage: Pairs of cage mates put in a cage with soiled bedding from other animals for 1 hr. Wet Cage: Pairs of cage mates put in an empty cage containing 1 cm of room-temperature water at the bottom for 1 hr, without access to food or water. Social isolation: 12 hr of isolation beginning at lights off without food and water, followed by 1 hr of water deprivation in the home cage in the morning; Wet bedding: Just before lights off, 400 ml water at room temperature was poured onto the bedding of the home cage. Animals were left for 13 hr beginning at lights off on damp bedding and given a clean cage at the end. Body weights were measured both on Day 1 and on the day following termination of CUS.

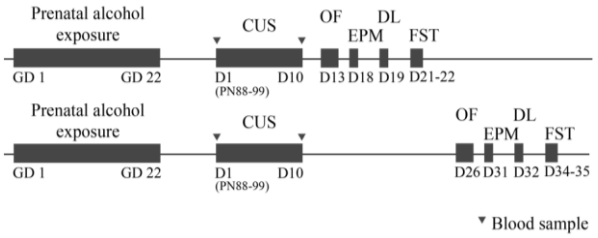

Figure 2.1 Experimental design.
GD = gestational day; D = day; PN = postnatal day; OF = open field test; EPM = elevated plus maze; DL = dark-light emergence test; FST = forced swim test.

2.2.4 Behavioral testing

Behavioral testing began 1 day or 14 days after the end of CUS (CUS-1 and CUS-14, respectively). Habituation to the testing room occurred 24 hr prior to each behavioral test. All animals were assessed on consecutive days, with a one-day break between tests, in the open field, elevated plus maze, light-dark emergence, and forced swim tests (**Figure 2.1**). This paper will focus on the behavior from the forced swim test.

The forced swim test apparatus was a transparent Plexiglas cylinder (20 cm diameter, 60.5 cm height). The cylinders were filled with 25 ± 1^{o}C water to a 44.5 cm depth to prevent animals' tails from touching the bottom of the tank (Detke et al., 1995). On Day 1, animals were placed in the cylinders for 15 min to experience the fact that escape was impossible, and on Day 2, they were tested for 5 min. Activity on both days were recorded by a video camera placed directly facing the cylinder for later scoring. Animals were towel-dried after each exposure and returned to preheated home cages. Duration of immobility, swimming, and climbing were measured, following the criteria of Armario et al. (Armario et al., 1988); i.e., immobility is when the rat performs the minimum movement necessary to stay afloat, swimming is when the rat moves

horizontally around in the cylinder, and climbing is when the forepaws actively break the surface of the water as the rat attempts to climb against the walls.

Testing occurred under dim lighting with white noise (30 dB) in the background to dampen random noise during the light phase of the circadian cycle. Behaviors in the forced swim test were recorded and scored using Observer 5.0 software (Noldus, Wageningen, The Netherlands). All behaviors were analyzed by an observer blind to the prenatal treatment and CUS conditions. For the forced swim test, n = 7-8/prenatal treatment/CUS condition/sex.

2.2.5 Tissue collection

Whole brains of the cohort of animals that was left undisturbed since being paired as cage mates were collected following decapitation under basal conditions. That is, the brains were collected in less than 3 min from touching their cages. For all other animals, whole brains were collected following decapitation 30 min after the onset of testing on Day 2, based on time-course studies demonstrating that *c-fos* mRNA expression generally peak at 30 min (Cullinan et al., 1995; Tang et al., 1997). Others have also successfully measured *c-fos* mRNA at the 30-min time point (da Costa et al., 1996; Kearns and Spencer, 2013; Ons et al., 2004; Torres et al., 1998). Whole brains were snap frozen on powdered dry ice and stored at -80°C. The brains of the 6 animals with the highest time immobile per prenatal treatment/CUS condition/sex were processed for *in situ* hybridization.

2.2.6 In situ hybridization

20μm coronal sections were collected on a cryostat at -16 ºC, mounted on slides (Superfrost slides, Fisher Scientific), and stored at –80ºC. For all brain areas, *n* = 4-6/prenatal treatment/CUS condition/sex. Samples from all animals for each brain region were processed simultaneously.

2.2.6.1 Probe and labeling

A ribonucleotide probe was used to detect *c-fos* mRNA in the medial prefrontal cortex (mPFC; anterior cingulate [Cg1], and prelimbic [PrL] and infralimbic [IL] cortices), amygdala (central, medial, lateral, and basal nuclei), paraventricular nucleus of the hypothalamus (medial parvocellular dorsal division [mpdPVN]), and the hippocampal formation (dentate gyrus [DG], CA3, CA1, and ventral subiculum). The rat *c-fos* ribonucleotide probe was prepared using a 2116 bp template provided by Dr. Victor Viau (Department of Cellular and Physiological Sciences, The University of British Columbia, Canada). The ribonucleotide probe was labeled with 35S-UTP (Amersham Biosciences, NJ, USA) using Polymerase T7 and Promega Riboprobe System (Promega Corporation, Madison, WI, USA). Micro Bio-Spin 30 Columns (Bio-Rad, CA, USA) was used to purify all probes. Oxidation was prevented by the addition of 1M of DTT.

2.2.6.2 Hybridization

Thawed slides (20 min) were fixed in formalin (30 min), and then pre-hybridized as previously described (Raineki et al., 2014). Briefly, slides underwent a series of washes, dehydrated through a series of increasing concentration of ethanol, delipidated in chloroform, and finally air-dried. After hybridization buffer mixed with the probe (activity of 1 x 10^6 cpm/slide) was applied, slides were covered with HybriSlips (Sigma-Aldrich, ON, Canada). Following incubation

overnight at 55°C in humidified chambers (75% formamide). HybriSlips were removed and slides were rinsed through a series of washes, dehydrated through a series of increasing concentration of ethanol, and air dried overnight.

Kodak BioMax MR autoradiography film was exposed to hybridized slides of the medial prefrontal cortex (mPFC) and hippocampal formation. Exposure times were as follows: 6 days for the mPFC, and 28 days for the hippocampal formation. The exposed autoradiographic films were developed using Kodak GBX developer and fixer.

Hybridized slides for the mpdPVN and the amygdala were dipped in Kodak NTB2 autoradiography emulsion (diluted 50:50 with distilled water) and exposed in desiccated, sealed, and light-tight boxes (4 °C) for 86 days in the PVN, and 134 days in the amygdala. Slides were developed using Kodak D19 developer and standard Kodak fixer, counterstained with 0.1% Walpole Toluidine Blue, and coverslipped with Permount (Fisher Scientific Ltd.).

2.2.7 Densitometric analysis

The autoradiographic films for mPFC and hippocampal formation were scanned and analyzed using Scion Image 4.0.3.2 (National Institutes of Health, USA) according to the Paxinos and Watson Stereotaxic Rat Brain Atlas, Fifth edition (Paxinos and Watson, 2004). Mean grey density levels were measured from Bregma 3.00 mm to 2.76 mm for the mPFC, and Bregma -4.80 mm to -5.28 mm for the hippocampal formation. Consistent with other studies in the literature, the Bregma range chosen for the hippocampal formation includes the ventral/temporal hippocampus which primarily relates to emotion and stress regulation (Abela et al., 2013; Bast et

al., 2009; Burton et al., 2009; Consolo et al., 1994; Fanselow and Dong, 2010; Ferbinteanu and McDonald, 2000; Strange et al., 2014). Two sections from the left hemisphere and two from the right hemisphere for each brain subregion were traced free-hand, the standard method in our laboratory (Raineki et al., 2014), resulting in a total of four measurements that were acquired from each subregion of each animal. Background was measured on the same side from the forceps minor for the mPFC and corpus callosum for the hippocampal formation. Corrected mean grey values were obtained by subtracting the background level from each of the four measurements and the four measurements from each animal were then averaged together for analysis.

In situ signals on the emulsion-dipped slides for PVN and amygdala were visualized with a Q-imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope. Images were captured under dark field illumination using Northern Eclipse v6.0 (Empix Imaging, Inc., Mississauga, ON, Canada) and analyzed with ImageJ 10.3 software (National Institutes of Health, USA) according to the Paxinos and Watson Stereotaxic Rat Brain Atlas, Fifth edition (Paxinos and Watson, 2004). Mean integrated density levels were measured from Bregma -2.64 mm to -3.00 mm for the amygdala, and Bregma -1.72 mm to -1.92 mm for the PVN. Two sections from the left hemisphere and two from the right hemisphere for each brain subregion were traced – with a fixed circle (diameter: 0.2 mm; scale 496 pixels/mm) for the mpdPVN or free-hand for the amygdala – for a total of four measurements that were acquired from each subregion of each animal. Background was measured, on the same side, from the adjacent internal capsule for the PVN and optic tract for the amygdala. Corrected mean

integrated density was obtained by subtracting the background from each of the four measurements and these were then averaged together for analysis.

2.2.8 Statistical Analyses

Univariate analyses of variance (ANOVAs) were performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software (IBM, Armonk, NY, USA) for the factors of prenatal treatment, CUS condition, and sex. Extreme outliers lower than 3 interquartile ranges below the first quartile or higher than 3 interquartile ranges above the third quartile were identified and removed (see Appendix C for details). Because main or interactive effects of sex were revealed for the behavioral measures (see Appendix D), all ANOVAs were then run separately for males and females. Significant main effects (e.g. effects of prenatal treatment independent of CUS, and effects of CUS independent of prenatal treatment) and interactions (prenatal treatment x CUS exposure) were then examined using *post-hoc* pairwise comparisons with Šídák correction. Differences were considered significant at $p \leq 0.05$.

Developmental data were analyzed using repeated-measures ANOVA (RM-ANOVA), with prenatal treatments (C, PF, PAE) as the between-subjects factor, and day of gestation or lactation for the dams and postnatal day for the offspring as the within-subjects factor. A separate one-way ANOVA was used to analyze pup body weights on PND 1. For all developmental data, degrees of freedom were adjusted using the Greenhouse-Geisser estimates of sphericity.

Change scores of body weight (i.e. post-CUS minus pre-CUS body weight of the same animal) were analyzed by ANOVAs for the factors of prenatal treatments and CUS exposure (Non-CUS, CUS).

Behavioral and *c-fos* mRNA data were analyzed using ANOVAs for the factors of prenatal treatment and CUS exposure (Non-CUS, CUS-1, CUS-14). Further analyses utilized planned comparisons to test the *a priori* hypotheses that: 1) PAE will alter depressive-like behavior and neural activation in response to behavioral testing; 2a) CUS will differentially alter depressive-like behavior and neural activity in PAE compared to C animals; and 2b) the differential effects of CUS on brain and behavior in PAE compared to C animals may depend on whether testing is immediate or delayed following termination of CUS exposure.

Further analyses using Constrained Principal Component Analysis (CPCA) were carried out to identify networks of brain regions associated with behavioral changes induced by the experimental conditions. CPCA is an exploratory multivariate technique that combines multivariate least-squares multiple regression and principal component analysis (PCA) to allow us to identify potential neural activation networks associated with interactive effects of PAE and CUS on depressive-like behavior. CPCA was performed in two steps, an *external analysis* and an *internal analysis*, and as for the univariate analyses, were run separately for males and females. In the first step, the *external analysis* uses multivariate multiple regression to separate the overall variance of the data (standardized using z-scores) with multiple dependent variables (i.e. forced swim test behavior on Day 2 and *c-fos* mRNA in multiple brain areas) into what can be predicted by the experimental manipulations (i.e., prenatal treatment and CUS exposure) from what

cannot. New independent variables were created using dummy coding. That is, for each rat, "1" was assigned for group membership and "0" for not (Hunter and Takane, 2002). Specifically, three dummy variables were used for prenatal treatment (C, PF, PAE) and three for stress condition (Non-CUS, CUS-1, and CUS-14); 9 dummy variables – 3x3 (3 dummy variables for prenatal treatment x 3 for stress condition) – were used to examine simple effects of treatment x CUS interaction. This external analysis produced four matrices of predicted scores that reflect the variance in brain and behavioral structures attributable to main effects of prenatal treatment, CUS condition, the overlap between prenatal treatment and CUS condition, and the interaction effect of prenatal treatment x CUS. To test the hypothesis that CUS will differentially alter behavior and neural activity in response to behavioral testing in PAE compared to C animals, only the matrix of the predicted scores of the variance attributable to prenatal treatment x CUS interaction effects was used for analysis in the second step of CPCA. This is the *internal analysis*, which is a PCA, an exploratory data reduction technique that transforms the predicted scores of our dependent variables (i.e., forced swim test behaviors on Day 2 and *c-fos* mRNA) into linear components that explain the maximum amount of total variance. Varimax with Kaiser normalization was used to rotate all PCA solutions – the *component loadings*. To determine the number of components to include, scree plots were inspected (Cattell, 1966; Cattell and Vogelmann, 1977). The component scores for each component were then correlated with the experimental condition to compute *predictor loadings* – which are correlation coefficients – and these were tested for reliable differences from 0 with a *t*-test. Previously published manuscripts provide a more detailed discussion of CPCA (Bodnar et al., 2017; Hunter and Takane, 2002; Lavigne et al., 2015, 2013; Liu et al., 2015; Metzak et al., 2011; Raineki et al., 2014; Sanford and Woodward, 2017; Takane, 2013; Takane and Hunter, 2001; Takane and Shibayama, 1991;

Woodward et al., 2013, 2006). CPCA was carried out using MATLAB R2016a (Mathworks, Natick, MA, USA).

2.3 Results

2.3.1 Developmental data

Blood alcohol levels were 134.1 ± 23.5 mg/dl in PAE dams and undetectable in PF and C dams. On GD 1, there were no differences in body weight among C, PF, and PAE dams, and all dams gained weight during pregnancy {TABLE 2.1; main effect of day ($F_{1.5,45.8} = 1317.968$, $p < 0.001$)}. However, over the course of gestation (GD 7, 14, and 21), C weighed more than both PF and PAE dams {treatment x day interaction ($F_{3.1,45.8} = 16.277$, $p < 0.001$)}. In addition, C weighed more than both PAE and PF dams on lactation day (LD) 1 {treatment x day interaction ($F_{4.3,67.4} = 14.945$, $p < 0.001$)}; however, by LD 8, body weights were no longer different among treatments.

Body weight of dams	Treatment during pregnancy		
	Control	Pair-fed	Alcohol-exposed
Pregnant dams (N)	10	11	13
Maternal death/illness (N)	0	0	0
Perinatal death	0.4 ± 0.2	0.36 ± 0.2	0.38 ± 0.2
Litter size	15.8 ± 0.8	14.7 ± 0.8	15.2 ± 0.7
Total litter mass	104.8 ± 4.4	101.3 ± 4.1	97.9 ± 3.8
Dam weight (g):			
GD 1	287.1 ± 3.5	289.3 ± 3.5	289.5 ± 3.1
GD 7	331.5 ± 4.2	307.5 ± 4.2[a]	305.8 ± 3.7[a]
GD 14	387.8 ± 6.4	354.5 ± 6.4[a]	347.5 ± 5.6[a]
GD 21	497.0 ± 9.2	445.0 ± 9.2[a]	434.8 ± 8.1[a]
LD 1	393.6 ± 7.5	367.0 ± 7.2[a]	349.0 ± 6.6[a]
LD 8	381.4 ± 7.4	369.1 ± 7.1	374.3 ± 6.5
LD 15	378.8 ± 7.4	368.5 ± 7.0	381.4 ± 6.5
LD 22	347.4 ± 6.0	336.3 ± 5.7	350.8 ± 5.3

Body weight of offspring	Prenatal treatment		
	Control	Pair-fed	PAE
MALES			
PND 1	6.9 ± 0.2	7.1 ± 0.2	6.6 ± 0.2
PND 8	17.7 ± 0.6	18.7 ± 0.6	18.0 ± 0.5
PND 15	33.9 ± 2.0	35.3 ± 1.9	33.9 ± 1.8
PND 22	56.1 ± 1.5	57.5 ± 1.4	59.3 ± 1.3
Pre-CUS	548.3 ± 11.2	553.8 ± 11.2	561.4 ± 11.2
FEMALES			
PND 1	6.7 ± 0.1	6.8 ± 0.1	6.4 ± 0.1
PND 8	17.1 ± 0.5	17.7 ± 0.5	17.4 ± 0.5
PND 15	33.0 ± 1.3	34.2 ± 1.2	35.2 ± 1.1
PND 22	54.9 ± 1.5	55.7 ± 1.4	56.7 ± 1.3
Pre-CUS	314.2 ± 6.5	311.1 ± 6.5	337.5 ± 6.5[b]

Table 2.1 Dam and offspring body weights (g, mean ± SEM).
[a] PAE = PF < C; [b] PAE > C = PF.

There were no effects of prenatal treatment on litter size, total litter mass, perinatal deaths, or birth weights **(TABLE 2.1)**.

As expected, both male and female pups gained weight from PND 1 to 22 {**TABLE 2.1**; main effect of day (males: $F_{1.8,54.4} = 1358.064$, $p < 0.001$; females: $F_{1.3,40.1} = 2988.417$, $p < 0.001$)}, and there were no differences in body weights among treatments during the preweaning period.

2.3.2 Body weight pre- and post-CUS

On Day 1 of CUS, body weights of adult male offspring did not differ by prenatal treatment. By contrast, adult PAE females weighed more than C and PF females {**TABLE 2.1**; main effect of prenatal treatment ($F_{2,69} = 4.995$, $p = 0.009$)}.

While all males gained weight over the 10-day period of CUS, CUS males gained less weight than non-CUS males {**FIGURE 2.2**; main effect of CUS exposure ($F_{1,66} = 135.729$, $p < 0.001$)}. By contrast, non-CUS females gained weight over the 10 days, while CUS females lost weight {**FIGURE 2.2B**; main effect of CUS exposure ($F_{1,66} = 23.368$, $p < 0.001$)}.

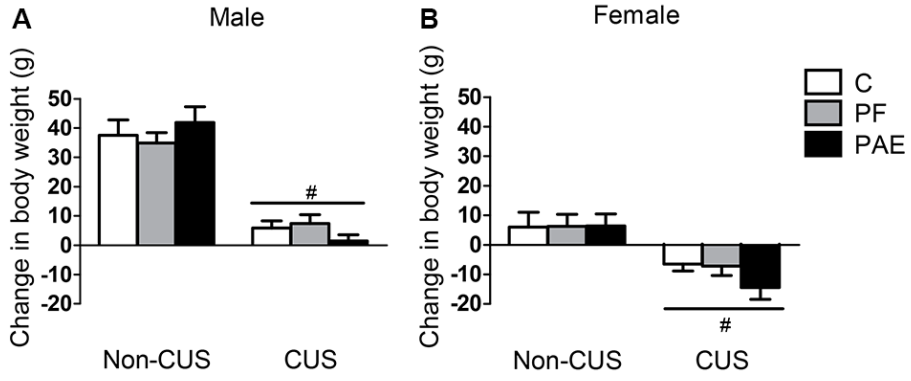

Figure 2.2 Change in body weight following CUS exposure.
Bars represent change scores (difference between pre- and post-CUS body weight; mean ± SEM) following the 10-day period of CUS in control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) males (**A**) and females (**B**). # indicates a significant main effect of CUS exposure, where animals exposed to CUS are different from non-CUS animals (non-CUS: *n* = 8/prenatal treatment/sex; CUS: *n* = 16/prenatal treatment/sex).

2.3.3 Forced swim test

2.3.3.1 Behavior on Day 1

In males, CUS decreased total time spent immobile in the forced swim test regardless of whether testing was immediate (CUS-1) or delayed (CUS-14) {**FIGURE 2.3A**; main effect of CUS ($F_{2,63}$ = 5.781, p = 0.005)}. Consequently, total time spent swimming in the forced swim test was increased in males under both CUS-1 and CUS-14 conditions compared to males in the non-CUS condition {**FIGURE 2.3B**; main effect of CUS ($F_{2,63}$ = 7.345, p = 0.001)}. Additionally, a *priori* analyses, based on our hypotheses, showed that CUS-1 PAE males spent less time immobile {Figure 2.3**A**; CUS-1 PAE < CUS-1 C ($F_{1,63}$ = 5.037, p = 0.028)} and more time swimming than CUS-1 C males {Figure 2.3**B**; CUS-1 PAE > CUS-1 C (p = 0.014)}. There were no effects of prenatal treatment or CUS on climbing in males. In females, there were no effects of prenatal treatment or CUS on any behavior.

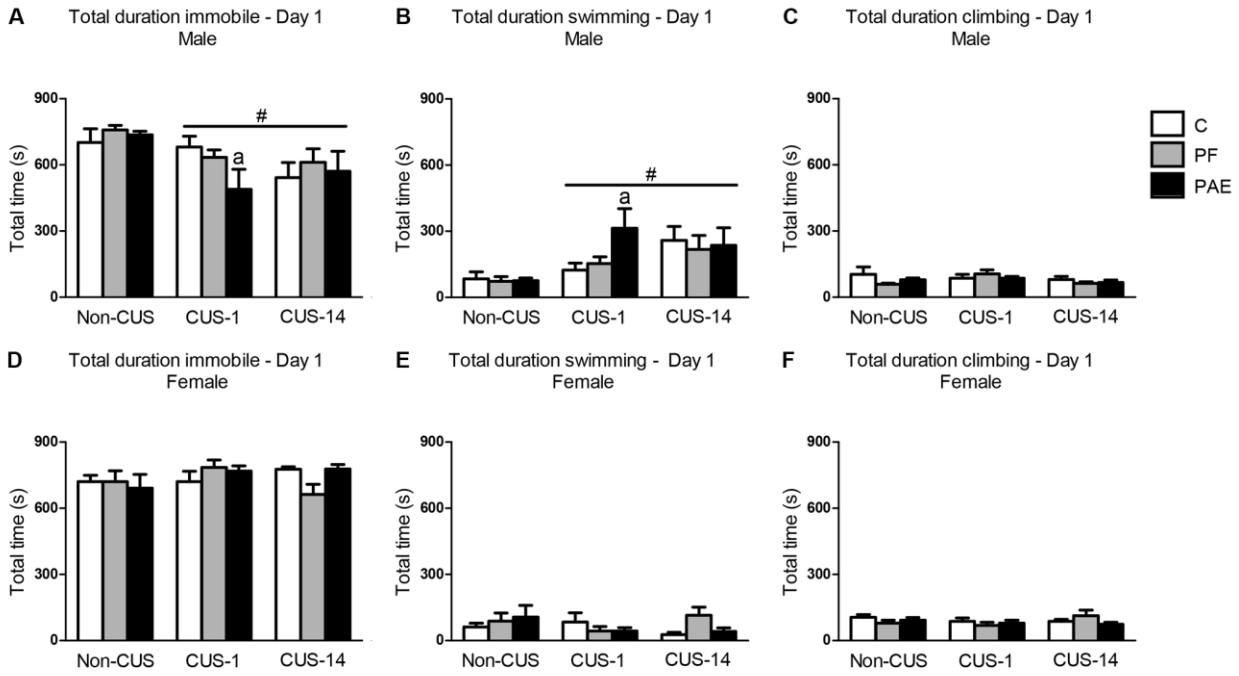

Figure 2.3 Immediate (CUS-1) and delayed (CUS-14) effects of CUS and prenatal alcohol exposure (PAE) on behaviors in the forced swim test on Day 1.
Bars represent the total time (mean ± SEM) control (C), pair-fed (PF), and PAE males and females spent immobile (**A,D**), swimming (**B,E**), and climbing (**C,F**) in the forced swim test on Day 1. [#] indicates a significant main effect of CUS: CUS-1 and CUS-14 are different from Non-CUS for **A,B**; [a] indicates that CUS-1 PAE is significantly different from CUS-1 C based on *a priori* comparisons (*n* = 7-8/prenatal treatment/CUS condition/sex).

2.3.3.2 Behavior on Day 2

In males, CUS exposure overall decreased total time spent immobile in the forced swim test {Figure 2.4**A**; main effect of CUS ($F_{2,63} = 13.412$, $p < 0.001$)}. Consequently, total duration of swimming and climbing were increased following CUS {**FIGURE 2.4B,C**; main effects of CUS exposure (swimming: $F_{2,63} = 9.356$, $p < 0.001$; climbing: $F_{2,63} = 3.369$, $p = 0.041$)}.

In comparison to males, all females in the CUS-1 condition showed an increase in total time spent immobile compared to non-CUS females {Figure 2.4**D**; main effect of CUS ($F_{2,61} = 4.451$, $p = 0.016$)}, and consequently, these females spent less total time swimming {Figure 2.4**E**; main effect of CUS ($F_{2,61} = 4.051$, $p = 0.022$)}. By contrast, in the CUS-14 condition, only PAE

females spent more total time immobile than C females {Figure 2.4**D**; *a priori* analysis: CUS-14 PAE > CUS-14 C ($F_{1,61}$ = 4.046, p = 0.049)}. CUS-14 PAE females also spent less total time climbing than their C and PF female counterparts, and CUS-14 PF females spent less total time climbing than their CUS-1 counterpart {treatment x CUS interaction (Figure 2.4**F**; $F_{4,61}$ = 3.450, p = 0.013)}.

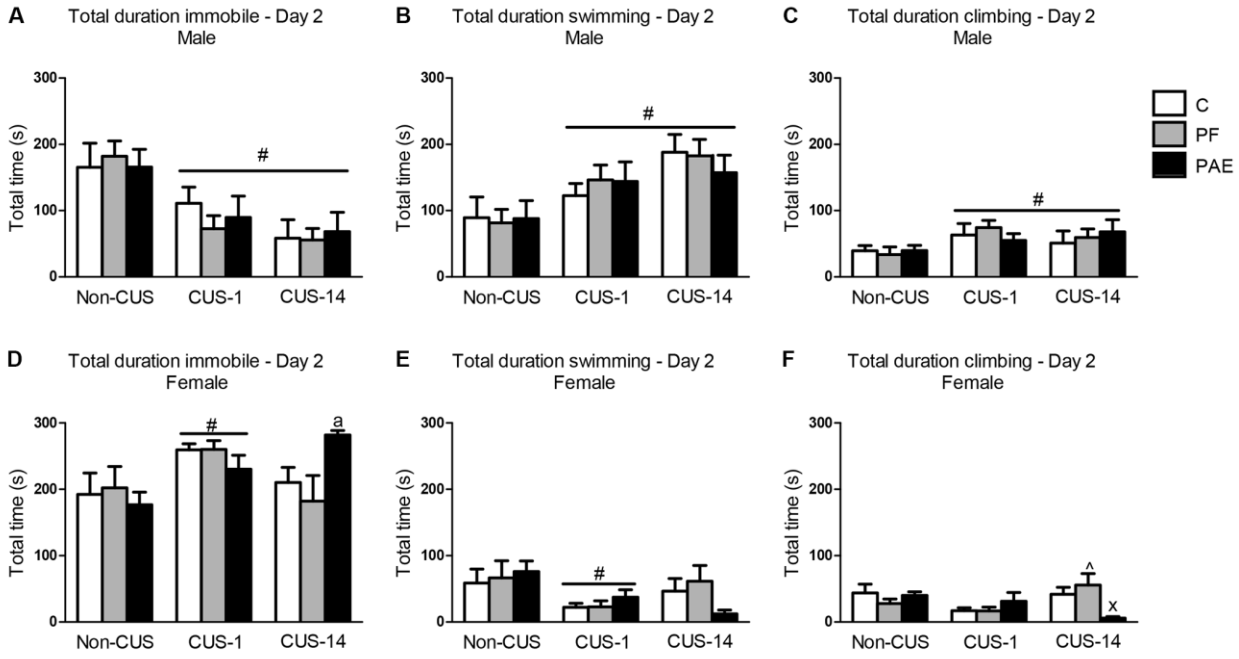

Figure 2.4 Immediate (CUS-1) and delayed (CUS-14) effects of CUS and prenatal alcohol exposure (PAE) on behaviors in the forced swim test on Day 2.
Bars represent the total time (mean ± SEM) control (C), pair-fed (PF), and PAE males and females spent immobile (**A,D**), swimming (**B,E**), and climbing (**C,F**) in the forced swim test on Day 2. [#] indicates a significant main effect of CUS: CUS-1 and CUS-14 are different from Non-CUS for **A-C**, and CUS-1 is different from Non-CUS for **D,E**; significant treatment x CUS interaction: [^] indicates that CUS-14 PF is different from CUS-1 PF, and [x] indicates that CUS-14 PAE is different from both CUS-14 C and CUS-14 PF; [a] indicates that CUS-14 PAE is significantly different from CUS-14 C based on *a priori* comparisons; (*n* = 7-8/prenatal treatment/CUS condition/sex).

2.3.4 *c-fos* mRNA expression

c-fos mRNA expression from brains of the cohort of animals collected under basal conditions was undetectable or very low such that measurements could not be reliably performed.

2.3.4.1 Medial prefrontal cortex

There were no effects of prenatal treatment or CUS exposure on *c-fos* mRNA expression in the mPFC in males (Figure 2.5**A,B,C**). By contrast, in the Cg1, PAE females overall had lower *c-fos* mRNA expression than their C counterparts {Figure 2.5**D**; main effect of prenatal treatment ($F_{2,46} = 3.335$, $p = 0.044$)}. As well, CUS-14 females overall had lower *c-fos* mRNA expression than both CUS-1 and Non-CUS females {Figure 2.5**D**; main effect of CUS ($F_{2,46} = 4.470$, $p = 0.017$)}. In the PrL, *c-fos* mRNA expression was lower in CUS-14 females compared to females in the CUS-1 and non-CUS conditions {Figure 2.5**E;** main effect of CUS ($F_{2,46} = 4.893$, $p = 0.012$)}. In the IL, *c-fos* mRNA expression was lower in CUS-14 than non-CUS females {Figure 2.5**F**; main effect of CUS ($F_{2,46} = 4.009$, $p = 0.025$)}.

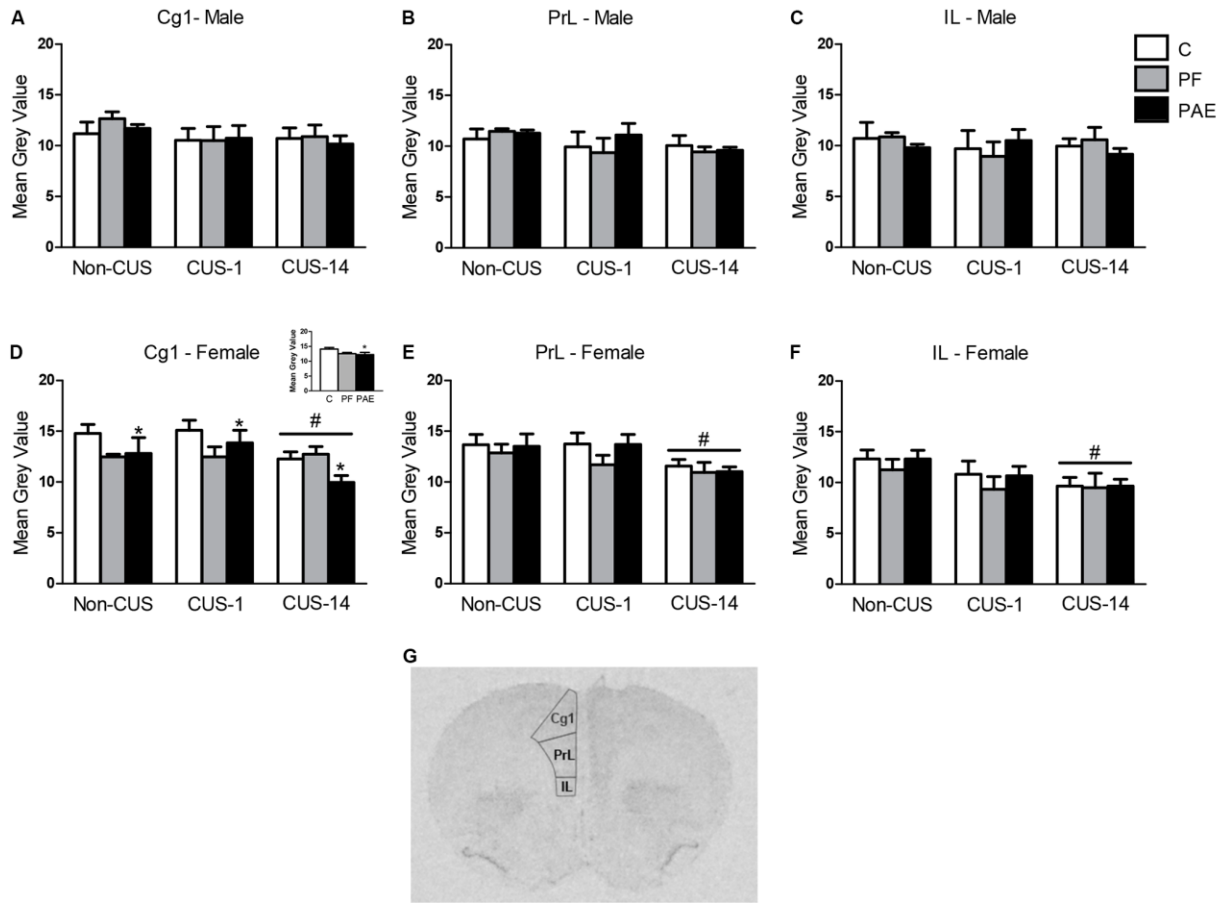

Figure 2.5 *c-fos* mRNA expression in the medial prefrontal cortex (mPFC) following Day 2 of the forced swim test in response to behavioral testing initiated 1- or 14-day post-CUS (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.
Bars represent mean grey value (mean ± SEM) of *c-fos* mRNA expression in the Cg1 (**A,D**), PrL (**B,E**), and IL (**C,F**). Free-hand delineations of the subregions are demonstrated in a representative image of an autoradiographic film of the mPFC (**G**). * indicates a significant main effect of prenatal treatment, where PAE is different from C – see inset graph in **D**; # indicates a significant main effect of CUS: CUS-14 is different from Non-CUS and CUS-1 for **D,E**; CUS-14 is different from non-CUS for **F**; (*n* = 5-7/prenatal treatment/CUS condition/sex).

2.3.4.2 Amygdala

In males, the ANOVA revealed no effects of prenatal treatment or CUS exposure for any of the amygdala nuclei (Figure 2.6**A-D)**. However, *a priori* analyses indicated that *c-fos* mRNA expression was lower in Non-CUS PAE males than their C counterparts in both the lateral and

central nuclei of the amygdala {Figure 2.6**A,C**; Non-CUS PAE < Non-CUS C (lateral: $F_{1,43}$ = 4.728, p = 0.035 and central: $F_{1,43}$ = 4.530, p = 0.039)}.

In females, there were no effects of prenatal treatment or CUS exposure on *c-fos* mRNA expression in any of the amygdala nuclei (Figure 2.6**E-H**).

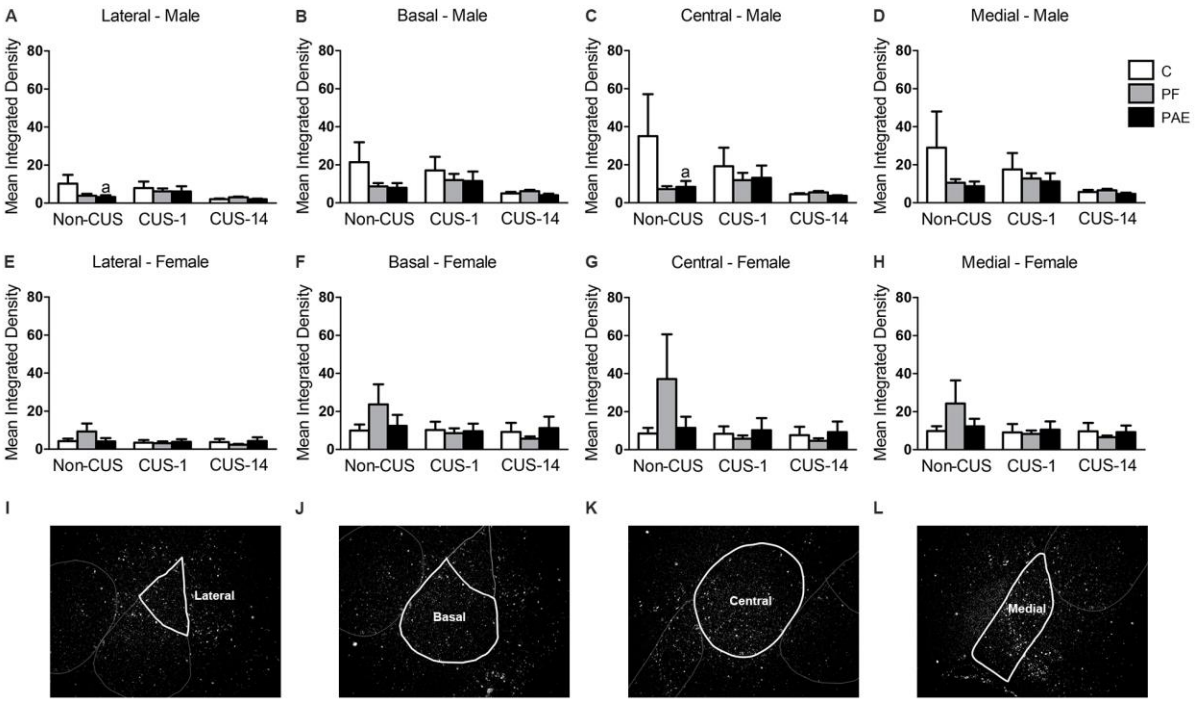

Figure 2.6 *c-fos* mRNA expression in the amygdala following Day 2 of the forced swim test in response to behavioral testing initiated 1- or 14-day post-CUS (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.
Bars represent mean integrated density (mean ± SEM) of *c-fos* mRNA expression in the lateral (**A,E**), basal (**B,F**), central (**C,G**), and medial (**D,H**) nuclei of the amygdala. Free-hand delineations of the lateral (**I**), basal (**J**), central (**K**), medial (**L**) nuclei are demonstrated in representative images of dark-field photomicrographs of a nuclear emulsion-dipped section of the amygdala. [a] indicates that non-CUS PAE is different from non-CUS C based on *a priori* comparisons (*n* = 5-6/prenatal treatment/CUS condition/sex).

2.3.4.3 Hippocampal formation

ANOVA revealed no effects of prenatal treatment or CUS exposure on the CA3, DG, or ventral subiculum in either males or females (Figure 2.7**A,B,D,E,F,H**). However, *a priori* analyses showed that CUS-14 PAE males had higher *c-fos* mRNA expression than their C counterparts in CA1 {Figure 2.7**C**; CUS-14 PAE > CUS-14 C ($F_{1,38} = 5.627$, $p = 0.023$)}; inspection of Figure 2.7**C** suggests that this was likely due to a directional (non-significant) decrease in CA1 activity in C, but not PAE, males in the CUS-14 compared to the non-CUS condition. There were no effects of prenatal treatment or CUS on CA1 in females (Figure 2.7**G**).

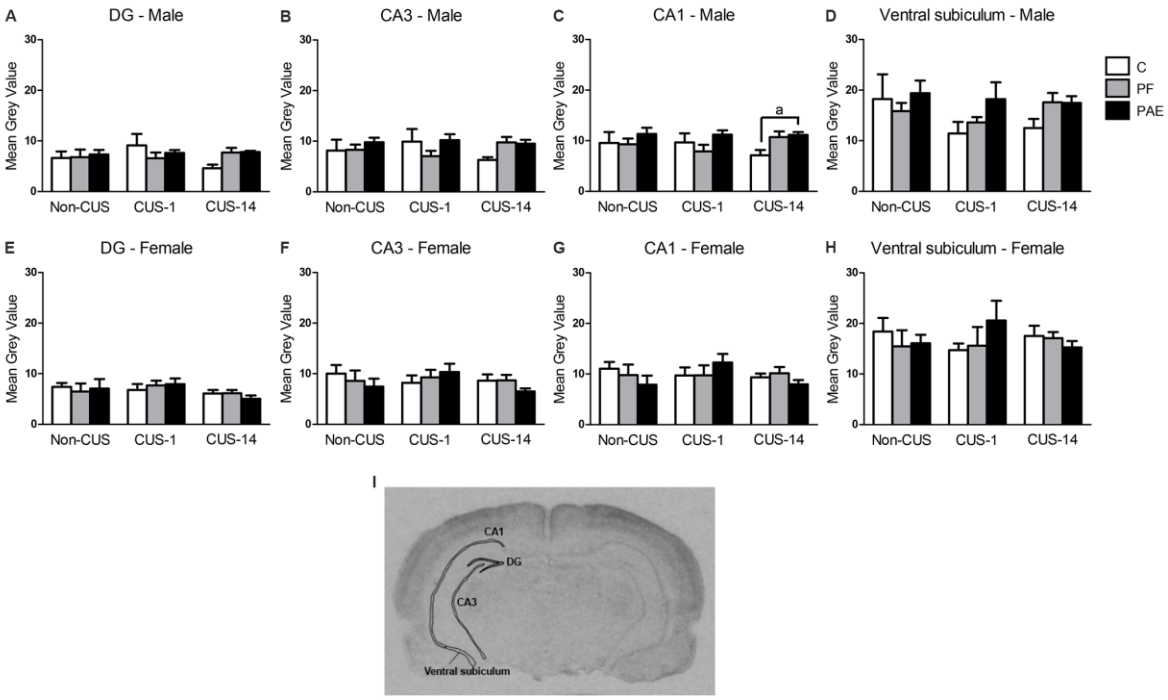

Figure 2.7 *c-fos* mRNA expression in the hippocampal formation following Day 2 of the forced swim test in response to behavioral testing initiated 1- or 14-day post-CUS (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol exposed (PAE) rats.
Bars represent mean grey value (mean ± SEM) of *c-fos* mRNA expression in the DG (**A,E**), CA3 (**B,F**), CA1 (**C,G**), and ventral subiculum (**D,H**). Free-hand delineations of the subregions are demonstrated in a representative image of an autoradiographic film of the hippocampal formation (**I**). [a] indicates that CUS-14 PAE is different from CUS-14 C rats based on *a priori* comparisons (*n* = 5-6/prenatal treatment/CUS condition/sex except n=4 for CUS-1 PAE in **A**; Non-CUS C, CUS-1 PAE in **B,C**; Non-CUS C, CUS-1 C, and CUS-1 PAE in **D**; and Non-CUS PF in **E-H**).

2.3.4.4 Hypothalamus

Neither prenatal treatment nor CUS exposure altered *c-fos* mRNA expression in the mpdPVN in males and females (Figure 2.8).

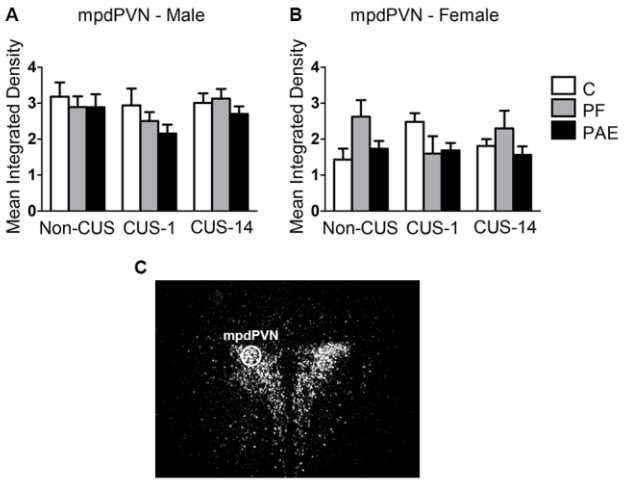

Figure 2.8 *c-fos* mRNA expression in the mpdPVN following Day 2 of the forced swim test in response to behavioral testing initiated 1- or 14-day post-CUS (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol exposed (PAE) rats.
Bars represent mean integrated density (mean ± SEM) of *c-fos* mRNA expression in males (**A**) and females (**B**). The mpdPVN was traced using a fixed circle as demonstrated in a representative image of a dark-field photomicrograph of a nuclear emulsion-dipped section of the PVN (C) (*n* = 5-6/prenatal treatment/CUS condition/sex).

2.3.5 Constrained principal component analysis (CPCA)

Note: The interpretation of the correlations between component scores and experimental group variables (prenatal treatment x CUS condition interaction) depends on the direction of component loadings (i.e. whether it is positive or negative). For example, when *c-fos* mRNA expression of a specific brain subregion positively loads on a component, a higher component score is associated with a higher *c-fos* mRNA expression. A subsequent positive correlation between the component score and the experimental group variable thus indicates that this experimental group is associated with higher *c-fos* mRNA expression from this subregion (i.e.

increased activation), whereas a subsequent negative correlation indicates decreased activation. By contrast, when a brain subregion negatively loads on a component, a higher component score is associated with lower *c-fos* mRNA expression. A subsequent positive correlation then indicates decreased activation, whereas a negative correlation indicates increased activation.

2.3.5.1 Males

The effects of prenatal treatment and CUS exposure combined accounted for 19.11% of the overall variance in brain and behavioral structures. Of that, 5.47% was due to the main effect of prenatal treatment independent of CUS, 13.58% was due to the main effect of CUS independent of prenatal treatment, and the remaining 0.06% was due to the overlap between prenatal treatment and CUS. The interaction between prenatal treatment and CUS accounted for an additional 5.37% of the overall variance in brain and behavioral structures. This variance constrained to the interaction between prenatal treatment and CUS was then further analyzed with a PCA, which revealed a four-component solution. The third and fourth components were not further analyzed because their component loadings were below 0.23, which was established as the cut-off point prior to analysis. The first component explained 44.71% of the predictable variance; it was defined as *Medial prefrontal cortex* because the three subregions of the mPFC (i.e. PrL, IL, and Cg1) showed the highest loadings, all in a negative direction. The second component explained 32.78% of the predictable variance, and was defined as *Hippocampal formation*, with the highest positive loadings from all subregions of the hippocampal formation: DG, CA3, CA1 and ventral subiculum. Behavioral measures did not load with brain subregions in either of these components **(**Table 2.2**)**.

Variables	*Medial prefrontal cortex*	*Hippocampal formation*
Cg1	**-0.23**	-0.04
PrL	**-0.33**	-0.08
IL	**-0.38**	0.09
Lateral amygdala	-0.10	0.04
Basal amygdala	-0.09	0.02
Central amygdala	-0.07	0.08
Medial amygdala	-0.08	-0.07
DG	0.07	**0.23**
CA3	-0.03	**0.25**
CA1	-0.09	**0.23**
Ventral subiculum	-0.10	**0.23**
mpdPVN	-0.01	0.07
Immobile duration (Day 2)	0.04	-0.02
Swimming duration (Day 2)	-0.03	0.06
Climbing duration (Day 2	-0.03	-0.08

Values ≥ 0.23 are set in bold.

Table 2.2 Component loadings for the predicted solution in males.

For the *Medial prefrontal cortex* network **(**Figure 2.9**A)**, correlations between component scores and experimental groups showed opposite patterns for C and PAE animals. For C, there was a negative correlation (increased activation) for the Non-CUS (r = -0.41, p = 0.005), a positive correlation (decreased activation) for the CUS-1 (r = 0.86, p < 0.001), and a negative correlation (increased activation) for the CUS-14 condition (r = -0.36, p = 0.015). By contrast, for PAE, there was a positive correlation (decreased activation) for the Non-CUS (r = 0.40, p = 0.006), a negative correlation (increased activation) for the CUS-1 (r = -0.81, p < 0.001), and a positive correlation (decreased activation) for the CUS-14 condition (r = 0.35, p = 0.020). These opposing patterns suggest that PAE by itself results in differential activation of the *Medial prefrontal cortex* compared to C, and that CUS overall differentially affects PAE and C males. There were no correlations for PF males, regardless of CUS condition.

For the *Hippocampal formation* network **(**Figure 2.9**B)**, correlations between component scores and experimental groups showed opposing patterns for C and PF males. For C, there was a positive correlation (increased activation) for both the Non-CUS ($r = 0.61$, $p < 0.001$) and CUS-1 ($r = 0.31$, $p = 0.039$) conditions, but a negative correlation (decreased activation) for the CUS-14 condition ($r = -0.84$, $p < 0.001$). By contrast, for PF, there was a negative correlation (decreased activation) for both the Non-CUS ($r = -0.36$, $p = 0.016$) and CUS-1 ($r = -0.52$, $p < 0.001$) conditions, but a positive correlation (increased activation) for the CUS-14 condition ($r = 0.83$, $p < 0.001$). These opposing patterns suggest that PF by itself results in differential recruitment of the *Hippocampal formation* network compared to C males, and CUS differentially affects PF and C males. There were no correlations for PAE males, regardless of CUS condition.

2.3.5.2 Females

The effects of prenatal treatment and CUS exposure combined accounted for 9.80% of the overall variance in brain and behavioral structures. Of that, 2.55% was due to the main effect of prenatal treatment independent of CUS, 7.18% was due to the main effect of CUS independent of prenatal treatment, and the remaining 0.06% was due to the overlap between prenatal treatment and CUS. The interaction between prenatal treatment and CUS accounted for an additional 10.72% of the overall variance in brain and behavioral structures. This variance constrained to the interaction between prenatal treatment and CUS was then further analyzed with a PCA, which revealed a four-component solution. The fourth component was not further analyzed because its component loadings were below 0.23. The first component explained 50.26% of the predictable variance; it was defined as *Forced swim test behavior + Cg1* because total duration of immobility, swimming, and climbing from Day 2 in the forced swim test, and

Cg1 activity together showed the highest loadings. Duration of immobility loaded negatively, while the other three variables loaded positively in this component. The second component explained 20.94% of the predictable variance, and was defined as the *Hippocampal formation*, with CA1, CA3, and ventral subiculum showing the highest positive loadings. The third component explained 20.12% of the predictable variance, and was defined as *PVN* as the mpdPVN alone loaded negatively on this component **(**Table 2.3**)**.

Variables	***Forced Swim test behavior + Cg1***	***Hippocampal formation***	***PVN***
Cg1	**0.26**	0.07	-0.15
PrL	0.00	0.08	-0.20
IL	-0.03	-0.07	-0.06
Lateral amygdala	-0.07	-0.07	0.13
Basal amygdala	-0.07	-0.14	0.09
Central amygdala	-0.02	-0.16	0.07
Medial amygdala	0.01	-0.11	0.13
DG	0.10	0.11	0.04
CA3	0.11	**0.26**	-0.01
CA1	0.10	**0.30**	-0.08
Ventral subiculum	0.15	**0.29**	-0.06
mpdPVN	0.01	0.03	**-0.44**
Immobile duration (Day 2)	**-0.51**	-0.04	-0.01
Swimming duration (Day 2)	**0.40**	0.06	0.05
Climbing duration (Day 2)	**0.51**	0.02	-0.06

Values ≥ 0.23 are set in bold.

Table 2.3 Component loadings for the predicted solution in females.

For the *Forced swim test behavior + Cg1* component **(**Figure 2.9**C)**, correlations between component scores and experimental groups showed opposing patterns for PF and PAE females. For PAE, there was a positive correlation (decreased immobility but increased swimming, climbing, and Cg1 activation) for both the Non-CUS ($r = 0.54$, $p < 0.001$) and CUS-1 ($r = 0.37$,

p = 0.013), and a negative correlation (increased immobility; decreased swimming, climbing, and Cg1 activation) for the CUS-14 condition (r = -0.87, p < 0.001), whereas for PF, there was a negative correlation (increased immobility; decreased swimming, climbing, and Cg1 activation) for the Non-CUS (r = 0.54, p <0.001), no correlation for the CUS-1, and a positive correlation (decreased immobility but increased swimming, climbing, and Cg1 activation) for the CUS-14 condition (r = 0.79, p < 0.001). These opposing patterns suggest that PAE and PF by themselves result in differential behavior and Cg1 activation compared to C, and both their behavior and Cg1 activity are differentially affected by CUS.

For the *Hippocampal formation* network **(**Figure 2.9**D)**, unlike males, correlations between component scores and experimental groups overall showed opposing patterns for C and PAE females. For C, there was a positive correlation (increased activation) for the Non-CUS (r = 0.35, p = 0.017), a negative correlation (decreased activation) for the CUS-1 (r = -0.69, p < 0.001), and a positive correlation (increased activation) for the CUS-14 condition (r = 0.34, p = 0.023). By contrast, for PAE, there was a negative correlation (decreased activation) for the Non-CUS (r = -0.68, p < 0.001), a positive correlation (increased activation) for the CUS-1 (r = 0.91, p < 0.001), and no correlation for the CUS-14 condition. As well, for PF females, there was a positive correlation (increased activation) for the Non-CUS (r = 0.39, p = 0.008), but no correlations for the CUS-1 or CUS-14 conditions in this component. These opposing patterns suggest that PAE by itself results in differential recruitment of the *Hippocampal formation* network compared to C, and CUS overall differentially affects PAE, PF, and C females.

For the *PVN* component **(**Figure 2.9**E)**, correlations between component scores and experimental groups showed opposing patterns for C and PF females. For C, there was a positive correlation (decreased activation) for the Non-CUS (r = 0.53, $p < 0.001$), a negative correlation (increased activation) for the CUS-1 (r = -0.67, $p < 0.001$), and no correlation for the CUS-14 C condition. By contrast, for PF, there was a negative correlation (increased activation) for the Non-CUS (r = -0.59, $p < 0.001$) and CUS-14 (r = -0.32, $p = 0.031$) conditions, but a positive correlation (decreased activation) for the CUS-1 condition (r = 0.90, $p < 0.001$). These patterns suggest that PF by itself causes differential recruitment of the *mpdPVN* compared to C and that CUS overall differentially affects PF and C females. There were no correlations for PAE females, regardless of CUS condition.

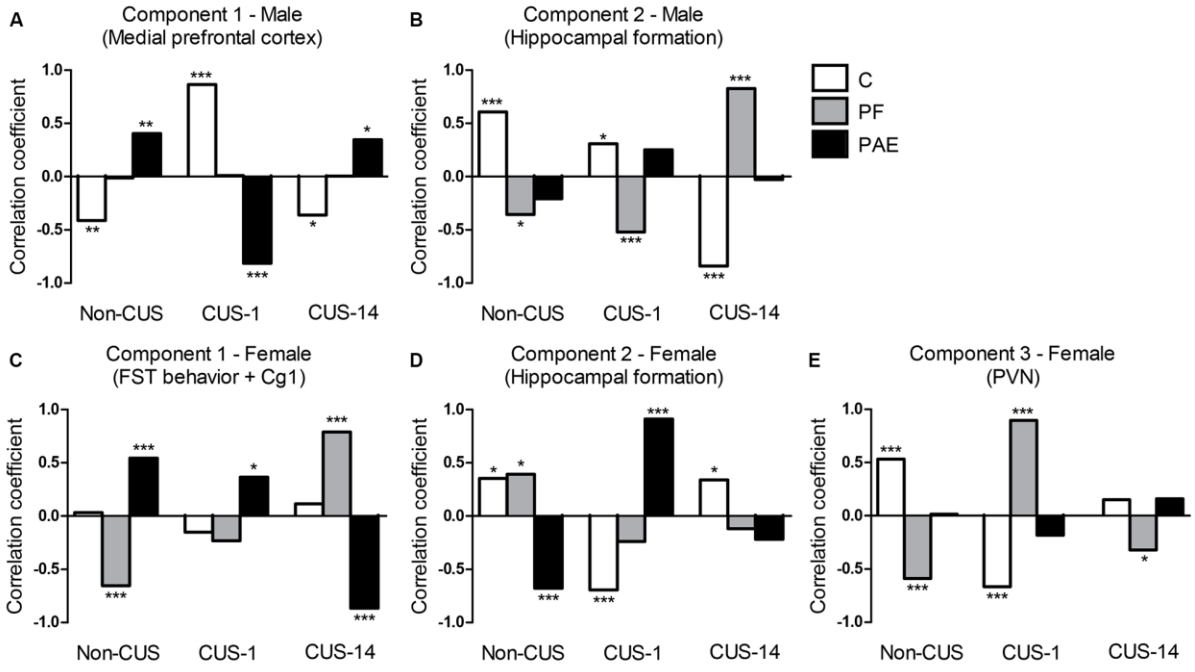

Figure 2.9 Correlations between the component scores and experimental groups extracted from the constrained principal component analysis.
Bars represent the correlation coefficients between component scores and experimental groups (i.e. simple effects of prenatal treatment x CUS exposure interaction) for males (**A,B**) and females (**C-E**). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. C, control; PF, pair-fed; and PAE, prenatal alcohol exposed. FST = forced swim test; PVN = paraventricular nucleus.

2.4 Discussion

Individuals prenatally exposed to alcohol are at a disproportionately higher risk than unexposed individuals of developing mental health problems, with a high incidence of depression reported. Brain areas implicated in the etiology of depression – including, but not limited to, the mPFC, amygdala, hypothalamus, and hippocampal formation – play a multiple role in behavioral, emotional, and stress regulation. The fine coordination of these interconnected brain regions supports appropriate regulation, and dysfunction of any one of these areas could result in maladaptive responses to stressors and/or the emergence of psychopathologies. Our results indicate that PAE and CUS interact to alter behavior in the forced swim test, as well as neural activation of several brain areas, with differential effects in males and females. Our CPCA results suggest that PAE alters the activation of neural and behavioral networks, and CUS differentially affects the networks of PAE, PF and C animals in a sex- and time-dependent manner. Overall, PAE-induced alterations in the neurocircuitry involved in behavioral, stress and emotional regulation, compounded with the impact of the later-life challenge of CUS, may underlie the increased risk for the development of depression in individuals prenatally exposed to alcohol.

2.4.1 PAE and CUS effects on body weight

Exposure to CUS in adulthood had an adverse impact on body weight regardless of prenatal treatment: males gained less weight while females lost weight compared to their non-CUS counterparts. These results are in line with findings from other studies showing that CUS exposure generally causes reduced weight gain in males (Bielajew et al., 2002; Cox et al., 2011) and are consistent with our previous data demonstrating that CUS may reduce weight *gain* in

males and induce weight *loss* in females (Hellemans et al., 2008; Uban et al., 2013). Changes in body weight following CUS also validate the effectiveness of our CUS paradigm. Importantly, our data suggest that males and females may be differentially responsive to the same CUS procedure.

2.4.2 PAE and CUS effects on forced swim test behavior

There was an overall but differential effect of CUS, independent of prenatal treatment, on behavior in the forced swim test in males and females, and the effect varied depending on whether testing occurred immediately or following a delay post-CUS. In males, CUS exposure decreased time immobile on both days of forced swim testing regardless of whether testing was immediate (CUS-1) or delayed (CUS-14), whereas in females, there was an increase in time immobile on Day 2, but only with immediate testing following CUS (CUS-1). The short-term effects of chronic stress on immobility has been variable in the literature: while most report an increase, a few have reported a decrease in immobility in the forced swim test (reviewed in (Willner, 2005)). These differences may be due to different stress procedures used (e.g. the duration of exposure and types of stressors), the parameters of testing (e.g. the temperature of the water, size of apparatus, and depth of water) (Detke and Lucki, 1996; Slattery and Cryan, 2012), as well as strain of the subjects (Bielajew et al., 2003). Sex of the animal may also influence forced swim test behavior, as we and others have also previously found that chronic stress induces differential alterations in immobility behavior and reward sensitivity to sucrose in males and females (Bielajew et al., 2003; Dalla et al., 2011; Hellemans et al., 2010a; Sachs et al., 2014). We now extend these findings to show that the effects of CUS can also be temporally dynamic depending on the sex of the animal.

Importantly, we found that PAE and CUS interact to differentially alter behavior of males and females in the forced swim test. In males, we found decreased time immobile in PAE compared to C males on Day 1 with immediate testing post-CUS (CUS-1), but no differences between groups on Day 2. By contrast, in females, CUS had no effects on PAE animals on Day 1 of the forced swim test, but had an adverse delayed effect on Day 2: specifically, CUS-14 PAE females showed an increase in time immobile compared to C females. Our previous study showed that PAE males exhibited lower immobility while PAE females exhibited higher immobility than their respective C counterparts in response to immediate testing following CUS, but these effects were observed on Day 2 of testing for males and on both Days 1 and 2 for females (Hellemans et al., 2010b). The difference in results between our previous and current studies may be due to the CUS paradigm used. Nevertheless, our findings suggest differential time-dependent alterations in forced swim test behavior in PAE males and females following CUS.

Immobility in the forced swim test has been suggested to represent "behavioral despair," which arises after realizing escape is impossible. This stems from the origin of the forced swim test as a preclinical screen for antidepressants; that is, antidepressants that are utilized in humans are found to decrease immobility in rats in this task (Borsini and Meli, 1988; Cryan et al., 2005). However, behaviors in the forced swim test can also be described as measures of active-coping strategies, with immobility representing a passive coping response (Commons et al., 2017; Slattery and Cryan, 2012). That CUS selectively increased immobility, whether it is a reflection of behavioral despair or passive coping, in PAE female rats is consistent with clinical literature suggesting that depression may be almost twice as common in women than men, possibly due to increased susceptibility to environmental factors, such as stress, that may trigger depression

(Altemus, 2006; Kessler, 2003; Weiss et al., 1999). On the other hand, the decrease in immobility in PAE males may suggest decreased behavioral despair or an increase in active coping. Regardless of interpretation of the meaning of the behavior in the forced swim test, our findings indicate that CUS differentially affected the behavioral response of PAE males and females compared to their control counterparts to the acute stress of forced swim test exposure.

2.4.3 PAE and CUS effects on neural activation

2.4.3.1 Medial prefrontal cortex

Anatomical and electrophysiological studies suggest that the rat mPFC is related to the primate anterior cingulate cortex (ACC) and dorsolateral PFC (Heidbreder and Groenewegen, 2003; Seamans et al., 2008). The rat mPFC is involved in various aspects of cognitive processing (e.g. working memory, attention, behavioral flexibility, response initiation, motor planning, and reward anticipation), as well as emotional regulation (Heidbreder and Groenewegen, 2003; Koenigs and Grafman, 2009; Seamans et al., 2008). In humans, hypofrontality in the PFC has been associated with depression, with hypoactivation observed in the pregenual ACC – the "affective subdivision" (Bush et al., 2000; Devinsky et al., 1995; Vogt et al., 1992) – and the dorsolateral PFC in individuals with depression (Baxter et al., 1989; Cohen et al., 1989; George et al., 1997; Hamilton et al., 2012; Ito et al., 1996; Martinot et al., 1990; Mayberg et al., 1999; Yazici et al., 1992). We found that in females but not males, PAE caused a reduction in *c-fos* mRNA expression in the Cg1 compared to C and PF, independent of CUS exposure. This PAE-induced hypoactivation is reminiscent of the observed hypofrontality in individuals with major depression and may therefore subserve an overall predisposition or vulnerability to develop depressive-like behavior.

CUS also resulted in an overall decrease in neural activation in the female Cg1, PrL, and IL, but only with delayed testing post-CUS, regardless of prenatal treatment. Our finding is temporally inconsistent with our behavioral data, but it is possible that while the behavioral consequence of CUS was more apparent immediately, changes in neural activation showed a delayed onset because of potential structural and neurobiological effects of stress that may possibly take time to occur. For example, levels of several neurotrophin-related signaling proteins that are involved with depression, which are decreased in the PFC and hippocampus of suicide patients (Dwivedi et al., 2009, 2003) and of rats following chronic swim stress exposure (Qi et al., 2008), show alterations in hippocampal expression at 2 weeks, but not 1 day following chronic corticosterone (Gourley et al., 2008b). Future studies may help to elucidate the intracellular mechanisms in the mPFC that may underlie the delayed effects of CUS on neural activation. Nevertheless, our findings suggest that PAE females may be more susceptible than C and PF females to the effects of CUS on neural activation such that given the same decrease in neural activity, depressive-like behavior increased in PAE but not C females.

2.4.3.2 Amygdala

The amygdala is well known for its role in emotional processing and regulation of the neuroendocrine stress response (Myers et al., 2012; Ulrich-Lai and Herman, 2009), and dysregulation of the amygdala has been associated with major depression (Drevets et al., 2008; Krishnan and Nestler, 2010; Nestler et al., 2002; Ressler and Mayberg, 2007). Sensory information from thalamic and cortical inputs are initially processed and integrated in the lateral nucleus (LeDoux et al., 1990; Li et al., 1996; McDonald, 1998; Szinyei et al., 2000), which then transfers integrated information, either directly or relayed through the basal nucleus (Pitkänen et

al., 1995; Savander et al., 1995), to the central nucleus – the major output nucleus of the amygdala. In the present study, we found no effects of PAE in females, but in males, PAE reduced *c-fos* mRNA expression in the lateral and central nuclei of the amygdala. Clinical studies find that in individuals with depression, the amygdala is often hyperactive in response to an emotional stimulus (Carlson et al., 2006; Drevets et al., 2008, 2002; Sheline et al., 2001). Therefore, a decrease in immobility in the forced swim test would be expected to accompany the PAE-induced decrease in neural activity; instead, we found that PAE males were not different from controls in the forced swim test, regardless of prior CUS. Although the changes in neural activation in the amygdala do not appear to associate with our behavioral findings, these findings suggest that the amygdala may receive (lateral nucleus) and relay (central nucleus) information differently in PAE compared to control males, which may subsequently alter their response to environmental cues.

2.4.3.3 Hippocampal formation

The hippocampal formation plays a major role in learning and memory, as well as in emotional and stress regulation (Fanselow and Dong, 2010), and clinically, has been suggested to play a role in depression (MacQueen and Frodl, 2011; Videbech and Ravnkilde, 2004). We found no differences among prenatal treatments for females, but in males, PAE rats had higher *c-fos* mRNA expression in the CA1 subregion relative to controls in response to delayed testing. Furthermore, inspection of **Figure 6C** suggests that the higher *c-fos* expression in PAE males may be due to the small differential decrease in CA1 activity in C and PAE males in the CUS-14 compared to the non-CUS condition, which is consistent with previous findings in PAE compared to C males in CA1 activity following stress (Raineki et al., 2014). Although we did not

observe behavioral differences in the forced swim test between C and PAE males, given that CA1 is a major output site of the hippocampus, projecting to over 50 areas (Cenquizca and Swanson, 2007), it is possible that we have unmasked a potential deficit in PAE compared to C males in being able to appropriately modulate hippocampal activity following CUS exposure.

2.4.3.4 Paraventricular nucleus of the hypothalamus

The mpdPVN integrates stress-related input from, but not exclusive to, the mPFC, amygdala and hippocampal formation to mount an appropriate HPA response to stress (Adhikari et al., 2010; Myers et al., 2012). We found that both male and female PAE animals showed neural activity in the PVN comparable to controls regardless of CUS history. These findings are consistent with those from our previous study in which no differences in *c-fos* mRNA expression in the mpdPVN were found among prenatal treatment groups, regardless of CUS exposure (Raineki et al., 2014). However, PAE has previously been shown to increase neural activation of the PVN compared to that in controls in response to stressors such as footshock (Lee et al., 2000). It has been suggested that the magnitude and time course of activation may be stressor-dependent (Cullinan et al., 1995; Duncan et al., 1996). While we observed no differences among prenatal treatment groups following forced swim test, regardless of CUS exposure, the possibility that the time course of activation following forced swim test may be different from that of footshock, or that PAE may alter the pattern of activation cannot be ruled out. Further investigations are needed.

2.4.4 Constrained principal component analysis (CPCA)

Extending our statistical analysis with CPCA allowed us to move beyond examination of individual brain areas and identify potential neural activation networks associated with interactive effects of PAE and CUS, on forced swim test behavior. CPCA highlighted three important characteristics of our data. First, we found different networks for males and females. Importantly, while forced swim test behaviors did not load with either brain network in males, forced swim test behaviors loaded along with Cg1 in the first component in females. These CPCA findings suggest that PAE and CUS may have greater impacts on forced swim test behavior in females, and emphasize that interactive effects of PAE and CUS are sex dependent, underscoring the importance of examining sex differences in outcome.

Second, CPCA revealed altered activation of brain subregions (e.g. mPFC, hippocampal formation, and mpdPVN) that the univariate analyses did not readily expose. Furthermore, it showed that the amygdala was not recruited in any of the networks, suggesting that it is not uniquely affected by our experimental conditions in either males or females. Identification of these effects was possible because the external analysis step of CPCA essentially removed irrelevant noise, permitting us to focus on the portion of the overall variance that is predictable from the interaction between prenatal treatment and CUS condition.

Third, the neural activation patterns for each sex were strikingly different among C, PF, and PAE animals. In males, PAE and C showed opposite patterns of activation for the *Medial prefrontal cortex* but not the *Hippocampal formation* network, suggesting that the functionality of this network is fundamentally altered by PAE. Moreover, CUS exposure differentially altered the

activity of this network in PAE and C males. Given the role of the mPFC in cognitive processing and reappraisal/suppression of negative emotion (Koenigs and Grafman, 2009), the differential activation of the *Medial prefrontal cortex* network in PAE and C males following CUS may confer a predisposition to develop symptoms of stress-related disorders, such as depression, beyond the "behavioral despair" measured in the present study.

Interestingly, PAE females differed from PF females in their pattern of activation of the *Forced swim test behavior + Cg1* network, suggesting that the effects of PAE are not simply due to the effects of reduced food intake and the concomitant effects of that procedure, but are unique to alcohol exposure. PAE females also differed from C females for the *Hippocampal formation* network, suggesting that the functionality of this network is fundamentally altered by PAE. Furthermore, CUS exposure differentially altered the activity of both networks in PAE, PF, and C females depending on whether testing occurred immediately or following a delay. Overall, our findings indicate that PAE females engage neural strategies different from those of both PF and C animals. That differential effects of CUS on PAE, PF, and C animals depended on the timing of testing suggests that certain effects may take time to appear, while some effects may be transient.

2.4.5 Pair-feeding

In general, we found no effects of PF in the individual behavioral and brain measures. However, CPCA revealed striking differences in the pattern of neural network activation in PF compared to both controls and PAE. Opposing patterns of *Hippocampal formation* network activation were found for PF and C males, opposing patterns of *Forced swim test behavior + Cg1* network

activation were found for PF and PAE females, and opposing patterns of *PVN* network activation were found for PF and C females. The PF group, which receives a reduced ration of a nutritionally optimal diet matched in amount to that of an alcohol-consuming partner (Weinberg, 1985, 1984), is used to control for the effects of typical reduction in consumption of diets containing alcohol. However, as pair-feeding cannot control for the nutritional impacts of alcohol, such as effects on nutrients absorption and utilization, it is an imperfect control group. Additionally, these animals likely experience a component of prenatal stress because their daily rations, which are less than would be presented if given *ad libitum* access, are typically consumed within hours of food presentation, resulting in food deprivation and likely hunger until feeding the next day. Therefore, the behavioral, physiological, and neurological effects of PF on offspring represent, at least partially, the combined prenatal impact of mild stress and a reduced food ration. Interestingly, we previously found that PAE and PF dams differed in their maternal behavior, including nursing frequency, as well as off-nest, self-directed, and negative behaviors, but no differences in arched-back nursing, and licking and grooming behavior (Workman et al., 2015). Thus, it is possible that some of the differences in PAE and PF offspring may be mediated by different mother-infant interactions (Workman et al., 2015). Our CPCA findings that PF animals were in many ways different from both their C and, more importantly, PAE counterparts, provide support that PF is a treatment in itself and PAE effects on behavior and brain are not simply due to the effects of reduced food intake, but unique to the effects of alcohol exposure during prenatal development.

2.4.6 Summary and implications

The present results build on and expand existing knowledge of the impact of PAE on neural regulation of behavior, stress and emotion that may underlie increased vulnerability to psychopathologies such as depression. The sex-dependent PAE-induced changes identified may also underlie the stress dysregulation that is observed following PAE, which in itself can contribute to psychopathologies. We also identified several effects of CUS that may have a delayed onset, underscoring the value of studying time-dependent effects of CUS in providing a more comprehensive and clinically relevant understanding of the effects of chronic stress. Importantly, the use of CPCA allowed us to go beyond simple assessments of changes in individual brain areas, and proved a powerful tool for identifying sex-dependent changes in functional networks in PAE animals compared to controls, and the differential recruitment of these networks among prenatal treatment groups following CUS exposure, thus allowing us to see a bigger picture in addition to discrete examinations of individual brain areas. By examining PAE- and CUS-induced differences in individual measures of depressive-like behavior and neural activity along with a network approach, we provide a more complete representation of the adverse effects of the interaction between PAE and CUS on brain and behavioral outcome, as well as insight into the pathophysiology of depression. Taken together, our findings suggest that PAE, in interaction with later life stress, results in sex-dependent dysregulation of the neurocircuitry that subserves behavioral, emotional and stress regulation. In turn, this dysregulation may ultimately contribute to an increased vulnerability to psychopathologies, such as depression, that are often observed in individuals prenatally exposed to alcohol.

Chapter 3: Interactive effects of prenatal alcohol exposure and chronic stress in adulthood on anxiety-like behavior and central stress-related receptor mRNA expression: Sex- and time-dependent effects

3.1 Introduction

Children and adults prenatally exposed to alcohol show higher rates of mental health problems than unexposed individuals, with depression and anxiety being among the more commonly encountered disorders (Famy et al., 1998; O'Connor and Paley, 2009; Pei et al., 2011). Previous studies in rats showed that prenatal alcohol exposure (PAE) can indeed increase depressive- and anxiety-like behavior in adulthood (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Varlinskaya and Mooney, 2014; Wilcoxon et al., 2005). However, depression and anxiety are often observed in the context of stress and/or a dysregulated stress response system (the hypothalamic-pituitary-adrenal [HPA] axis) (Jacobson, 2014; Nestler et al., 2002). PAE can often result in HPA axis dysregulation. Data from several clinical studies demonstrate higher basal and stress cortisol levels, and studies using animal models extend and support the clinical findings, reporting increased HPA activation and/or a delayed return to basal levels, as well as altered central HPA regulation in PAE compared to control offspring (reviewed in (Hellemans et al., 2010a)). Therefore, HPA dysregulation induced by PAE may predispose these individuals to an increased vulnerability to stress-related disorders such as anxiety or depression following stress over the life course. This would have important clinical relevance because individuals prenatally exposed to alcohol are more likely to encounter stressful environments/experiences during their lifetimes (reviewed in (Hellemans et al., 2010a)). In support of this, we have previously found that chronic unpredictable stress (CUS) in adulthood increased anxiety- and

depressive-like behavior in PAE rats compared to controls, with differential effects in male and female offspring (Hellemans et al., 2010a).

The corticotropin-releasing hormone receptor type 1 (CRHR1), mineralocorticoid receptor (MR), and glucocorticoid receptor (GR) are key in regulating HPA activity. CRHR1 is one of the receptors that mediates the neuroregulatory effects of CRH, whereas MR and GR mediate the effects of glucocorticoids. CRHR1, activated by CRH, can both facilitate and depress neurotransmission (Gallagher et al., 2008). This receptor is widely expressed in the brain, including limbic regions such as the medial prefrontal cortex (mPFC), amygdala, and hippocampal formation (Henckens et al., 2016), and it has different roles in modulating HPA activity and behavior depending on where it is expressed. Cytosolic MR has a high affinity for glucocorticoids and is responsive primarily when glucocorticoids are at low levels, such as during the circadian trough or under non-stressed conditions. Generally, the MR is implicated in regulating basal HPA tone. However, membrane-bound MR, which has a lower affinity for glucocorticoids than cytosolic MR, may also rapidly influence HPA activity following stress through non-genomic mechanisms (ter Heegde et al., 2015). In contrast, the lower-affinity GR is activated when glucocorticoid hormones are at intermediate to high levels (e.g. during the glucocorticoid ultradian/circadian peak or in response to stress) (Reul and de Kloet, 1985). GRs appear to be involved primarily in mediating feedforward/feedback regulation of the stress response (Herman et al., 2016). Importantly, overexpression of CRH, mediated through CRHR1, as well as dysregulation of MR and GR have been suggested to underlie psychopathology such as depression and anxiety (Binder and Nemeroff, 2010; Holsboer and Ising, 2010, 2008; Inda et al., 2017; Joëls and de Kloet, 2017; Veenit et al., 2014).

We and others have shown previously that PAE has widespread effects on these stress-related receptors in the brain. Specifically, PAE increases CRH mRNA expression in the central nucleus of the amygdala in adult males and females (Lan et al., 2015), and differentially decreases CRHR1 mRNA expression in the mPFC, amygdala, hippocampus, and pituitary of adult males and females (Glavas et al., 2007; Raineki et al., 2018, 2016). CRHR1 mRNA and protein expression are also decreased in the hippocampus of adolescent PAE males (Caldwell et al., 2015; Raineki et al., 2018). Additionally, PAE decreases MR mRNA expression in the adult female hippocampus (Sliwowska et al., 2008; Uban et al., 2013), and alters hippocampal MR mRNA expression in adolescent male and GR mRNA expression in adolescent female rats (Raineki et al., 2018). PAE-induced dysregulation of these key receptor systems may therefore predispose individuals to the adverse effects of stress over the life course and increase vulnerability to stress-related disorders such as depression and anxiety. In support of this, we have found that chronic stress during adolescence may further alter mRNA expression of these stress-related receptors and anxiety-like behavior in PAE compared to C animals, and may do so in a sex-dependent manner (Raineki et al., 2018, 2016). As well, we recently showed that PAE and chronic stress in adulthood interact to result in sex- and time-dependent dysregulation of the neurocircuitry underlying behavioral, emotional and stress regulation, as well as alterations in depressive-like (forced swim test) behavior (Lam et al., 2018b [**Chapter 2**]). However, less is known about whether PAE may interact with chronic stress in adulthood to further impact MR, GR, and CRHR1 expression, and the implications of this interaction for anxiety-like behavior.

The present study aimed to determine the independent and interactive effects of PAE and adult CUS on anxiety-like behavior and receptor systems (MR, GR, and CRHR1) underlying stress

and emotional regulation. We also examined whether exposure to CUS results in immediate or delayed effects, because animal studies have shown differential changes in behavior and brain depending on whether there was a recovery period between chronic stress or corticosterone exposure and testing (Gourley and Taylor, 2009; Matuszewich et al., 2007). Anxiety-like behaviors of male and female rats were evaluated using the open field, elevated plus maze, and dark-light emergence tests. CRHR1, MR, and GR mRNA expression were assessed in the mPFC, amygdala, and hippocampal formation, brain areas key to both stress and emotional regulation.

3.2 Materials and methods

3.2.1 Animals and breeding

All animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, the Canadian Council on Animal Care, and approved by the University of British Columbia Animal Care Committee. Adult virgin male (275-300g) and female (265-300g) Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, PQ, Canada). Rats, pair-housed and given *ad libitum* access to standard laboratory chow and water, habituated to the facility for a 7-10-day period, which was maintained on a 12:12 hr light/dark cycle (lights on at 07:00 h) and temperature set at 21±1°C. For breeding, males were paired with a female and the presence of sperm in vaginal lavage samples taken every morning at 08:00 hr indicated day 1 of gestation (GD 1).

3.2.2 Diets and feeding

On GD 1, females were singly housed and randomly assigned to one of three treatment groups: 1) alcohol-fed (PAE; n=13), receiving a liquid ethanol diet with 36% ethanol-derived calories,

6.7% v/v; 2); 2) pair-fed (PF; n=11), receiving a liquid control diet with maltose-dextrin isocalorically substituted for ethanol, in an amount consumed by a PAE partner (g/kg body weight/GD); and 3) *ad libitum*-fed control (C; n=10), receiving a pelleted control diet. All diet formulations provide optimal nutrition during pregnancy (Weinberg/Keiver High Protein Ethanol [#710324] and Control [#710109] liquid diets, and Weinberg/Keiver High Protein Pelleted Control Diet [#710109] were prepared by Dyets, Inc., Bethlehem, PA, USA) (Lan et al., 2006). Fresh diets were presented daily at 1 hr prior to lights off to minimize shifts in the maternal corticosterone circadian rhythm (Gallo and Weinberg, 1981). At the same time, volume of liquid diet consumed since the previous night was recorded. All groups also received *ad libitum* access to water. To facilitate the transition into a full liquid ethanol diet beginning on GD 3 until GD 21, liquid ethanol diets were introduced gradually: on GD 1, rats were given a 1:2 ratio of liquid ethanol to liquid control diet; on GD 2, they were given a 2:1 ratio. Beginning on GD 22 and throughout lactation, dams received a 19% protein laboratory chow (Teklad Global #2019) and water.

On GD17, blood samples from the tail vein were collected 3 hr after lights off from a subset of C, PF, and PAE dams (n=3 each) and blood alcohol levels were determined using an assay from Pointe Scientific Inc. (Canton, MI, USA); PAE dams had BALs of 134.1 ± 23.5 mg/dl.

On the day of birth (postnatal day 1, PND 1), pups were weighed, and litters randomly culled to 12 (6 males, 6 females when possible). If necessary to maintain litter size, pups from the same prenatal treatment born on the same day were fostered into a litter. Dams and offspring were housed undisturbed except for weekly weighing and cage change. After weaning (PND 22), pups

were fed an 18% protein chow (Teklad Global #2018) and housed on standard non-ventilated racks. Pups of the same sex and prenatal treatment, but different litters born +/- 2 days, were pair-housed beginning PND 42 ± 2 day.

3.2.3 Chronic unpredictable stress (CUS) paradigm

Animals from each experimental group were randomly assigned to either stress (CUS) or no-stress (non-CUS) conditions. Only one male and one female offspring from each litter were assigned to any one condition. CUS involved 10 days of twice daily exposure to stressors at random time, with a minimum of 2 hr between, and in random order; all CUS rats experienced the same number of times of each stressor over the 10-day period.

Stressors used included: Platform: Animals individually placed on 20W x 20L x 90H cm transparent Plexiglas platforms for 20 min. Restraint: Animals individually restrained in PVC tubes (15 cm × 6 cm for females and 19 cm × 7 cm for males) with ventilation holes for 30 min. Soiled Cage: Pairs of cage mates placed in cages with soiled bedding from other animals for 1 hr. Wet Cage: Pairs of cage mates placed in empty cages containing 1 cm of room-temperature water at the bottom for 1 hr, without food and water. Social isolation: 12 hr of isolation beginning at lights off without food and water, followed by 1 hr of water deprivation in the home cage in the morning; Wet bedding: Bedding of home cages were wet with 400 ml of room-temperature water just before lights off. Animals were housed on wet bedding for 13 hr and given a clean cage at the end.

3.2.4 Blood sampling

Basal blood samples were collected via tail nick from both non-CUS and CUS animals on Day 1 of the CUS procedure and on the day following termination of CUS. Samples were collected on ice within 2 min of touching the cage to obtain a true basal measure, centrifuged within 60 min of collection at 3200 rpm for 10 min at 4°C, and the serum stored at -20°C until assayed.

3.2.5 Radioimmunoassays (RIA)

Total corticosterone levels (bound plus free) were measured using a modification of the ImmuChem^{TM} Corticosterone I^{125} RIA Kit (MP Biomedicals, Orangeburg, NY): all reagents and samples were halved, and assay was performed according to vendor instructions. The minimum detectable range was 7.7 ng/ml, and the inter- and intra-assay coefficients of variation were <7.2% and <10.3%, respectively.

3.2.6 Behavioral testing

Behavioral testing began 1 day or 14 days after the end of CUS (CUS-1 and CUS-14, respectively). Animal behaviors were assessed on consecutive days, with a one-day break between tests, in the open field, elevated plus maze, dark-light emergence, and forced swim tests. This paper focuses on the behaviors from the open field, elevated plus maze, and dark-light emergence tests. 24 hr prior to each behavioral test, all animals were habituated to the testing room for 20 min. Tests were performed as follows:

The open field apparatus was an 80 x 80 cm x 40 cm square arena enclosed by transparent Plexiglas walls. Animals were tested for 5 min per day over 3 consecutive days. Total distance

traveled (cm) in the field, as well as distance travelled, time spent, and frequency and latency of entries into the center zone on Day 1 of the test were analyzed to assess their unconditioned anxiety-like behaviors in response to a novel environment.

The elevated plus maze consisted of two open arms and two arms enclosed by 40 cm opaque walls (each arm is 50 x 10 cm, and the center area is 10 x 10 cm) elevated 40 cm above ground. Animals were tested once for 5 min. Time on the open arms as a percent of open and closed arms time, and frequency of closed arm entries were assessed.

The dark-light emergence test apparatus was a white Plexiglas arena (80 x 40 x 40 cm) with an enclosed black box (25 x 40 x 40 cm) placed at one end with an opening (10 x 10 cm) facing out into the lit area. Each rat was tested once for 10 min. Latency to enter and time spent in the light compartment were assessed.

All behavioral tests were done during the light phase of the circadian cycle, but occurred under dim lighting, except for the dark-light emergence test where 60 lux of lighting was used to illuminate the light compartment. White noise (30 dB) was used in the background to dampen random noise in the testing room. Behavior in the open field was recorded and scored using Ethovision v3.1 software (Noldus, Wageningen, The Netherlands). All other behaviors were recorded and scored using The Observer 5.0 software (Noldus, Wageningen, The Netherlands). All behaviors were analyzed by an observer blind to the prenatal treatment and CUS condition.

3.2.7 Tissue collection

Whole brains were collected via decapitation 30 min after the onset of testing on Day 2 of the forced swim test. Brains were collected and snap frozen on powdered dry ice and stored at -80°C.

3.2.8 In situ hybridization

20 μm coronal sections were mounted on slides (Superfrost slides, Fisher Scientific) in a cryostat at -16^{o}C, and stored at -80^{o}C. Ribonucleotide probes were used to detect CRHR1 and GR mRNA in the medial prefrontal cortex (mPFC; anterior cingulate [Cg1], and prelimbic [PrL] and infralimbic [IL] cortices), amygdala (central, medial, lateral, and basal nuclei), and the hippocampal formation (dentate gyrus [DG], CA3, CA1, and ventral subiculum). A cRNA ribonucleotide probe was also used to detect MR mRNA in the hippocampal formation. The rat CRHR1 ribonucleotide probe was prepared using a 1.3 kb template provided by Dr. Victor Viau (The University of British Columbia, Canada). The rat MR ribonucleotide probe was prepared using a 550 bp template (complementary to the coding region and 3' untranslated region of rat MR mRNA) from Dr. James Herman (University of Cincinnati, USA) (Herman et al., 1999). The rat GR ribonucleotide probe was prepared using a 456 bp template (complementary to the coding region and 3' untranslated region of rat GR mRNA), also provided by Dr. James Herman (Herman et al., 1999). The ribonucleotide probes were labeled with 35S-UTP (Amersham Biosciences, NJ, USA) using Polymerase T7 (CRHR1 and GR) or T3 (MR) and Promega Riboprobe System (Promega Corporation, Madison, WI, USA). All probes were purified using Micro Bio-Spin 30 Columns (Bio-Rad, CA, USA). 1M of DTT was added to prevent oxidation.

In situ hybridization was performed following previously described procedures (Raineki et al., 2016). Briefly, thawed slides underwent a series of washes, dehydrated through a graded series of ethanol, delipidated in chloroform, and finally air dried. After hybridization buffer mixed with the probe (activity of 1 x 10^6 cpm/slide) was applied, slides were covered with HybriSlips (Sigma-Aldrich, ON, Canada). Following incubation overnight at 55^{o}C in humidified chambers (75% formamide), HybriSlips were removed and slides were rinsed through a series of washes, dehydrated through a graded series of ethanol, and air dried overnight.

3.2.9 Densitometric analysis

Kodak BioMax MR autoradiography films were exposed to hybridized slides. The exposed autoradiography films were developed using Kodak GBX developer and fixer, and scanned and analyzed using Scion Image 4.0.3.2 (National Institutes of Health, USA) according to Paxinos and Watson (2004). Two sections each of the left and right subregions for each brain region per animal were traced freehand to determine mean grey density levels. Mean grey density levels were measured from Bregma 3.00 mm to 2.76 mm for the mPFC; Bregma -2.64 mm to -3.00 mm for the amygdala; and Bregma -4.80 mm to -5.28 mm for the hippocampal formation. Consistent with other studies in the literature, the Bregma range chosen for the hippocampal formation includes the ventral/temporal hippocampus which primarily relates to emotion and stress regulation (Abela et al., 2013; Bast et al., 2009; Burton et al., 2009; Consolo et al., 1994; Fanselow and Dong, 2010; Ferbinteanu and McDonald, 2000; Strange et al., 2014). Background was measured from white matter areas on the same side: forceps minor for the mPFC, internal capsule for the amygdala, and corpus callosum for the hippocampal formation. Corrected mean

grey values were obtained by subtracting the background level from each of the four measurements and the four measurements were then averaged together for analysis.

3.2.10 Statistical Analyses

Univariate analyses of variance (ANOVAs) were performed using IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software (IBM, Armonk, NY, USA). Extreme outliers lower than 3 interquartile ranges below the first quartile or higher than 3 interquartile ranges above the third quartile were identified and removed prior to statistical analyses (see Appendix C for details). Because main effects of sex were revealed for corticosterone results and key behavioral measures (see Appendix D), all ANOVAs were then run separately for males and females. Differences were considered significant at $p \leq 0.05$. Significant main effects and interactions were examined using *post hoc* pairwise comparisons with Šídák correction.

Pre-CUS corticosterone data were analyzed using a one-way ANOVA for the factor of prenatal treatment (C, PF, PAE); n = 23 for males, 24 for females. Post-CUS corticosterone levels (n = 7-8/treatment/CUS/sex) were analyzed using an ANOVA for the factors of prenatal treatment and CUS exposure (Non-CUS, CUS). Behavioral data (n = 7-8/ treatment/CUS/sex) and mRNA mean gray values (n = 4-6/treatment/CUS/sex) were analyzed using an ANOVA for the factors of prenatal treatment and CUS exposure (Non-CUS, CUS-1, CUS-14). Further analyses utilized planned comparisons to test the *a priori* hypotheses that: 1) PAE will alter anxiety-like behavior and MR, GR, and CRHR1 mRNA expression; and 2) CUS will differentially alter anxiety-like behavior and mRNA expression of MR, GR, and CRHR1 mRNA in PAE compared to control animals.

3.3 Results

3.3.1 Corticosterone

There were no differences among prenatal treatment groups for either males or females for pre- or post-CUS basal corticosterone levels. By contrast, CUS increased basal corticosterone levels [main effect of CUS ($F_{1,64} = 10.769$, $p = 0.002$)] in males but not females (Figure 3.1).

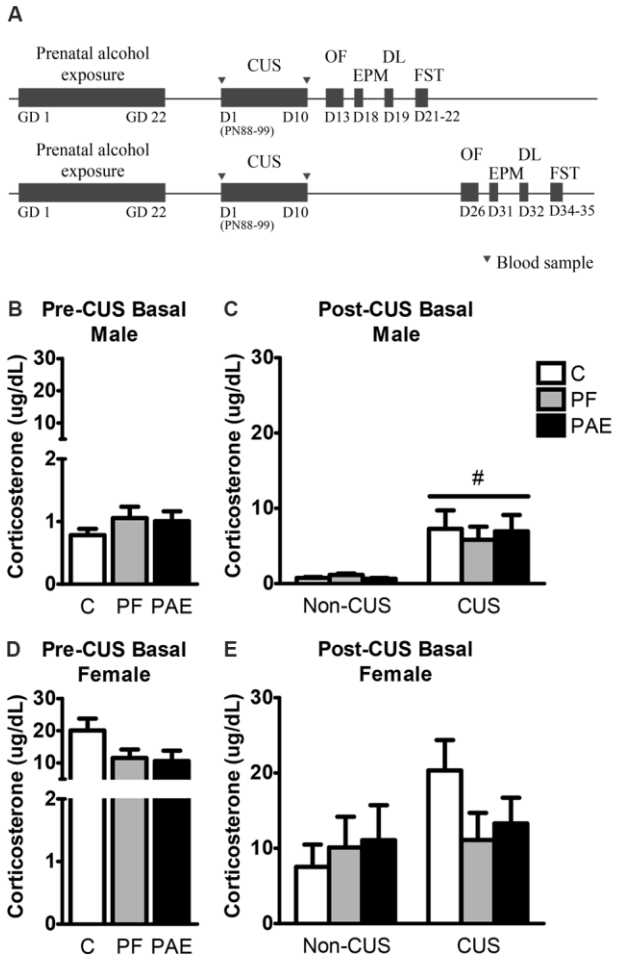

Figure 3.1 Experimental design (A) and basal corticosterone levels pre- (B,D) and post-CUS (C,E).
Bars represent the mean ± SEM (μg/dL) of basal corticosterone levels following prenatal alcohol exposure (PAE) and chronic unpredictable stress (CUS). # indicates a significant main effect of CUS (pre-CUS: n=23/prenatal treatment in males, n=24/prenatal treatment in females; post-CUS: n=7-8/prenatal treatment/sex for non-CUS, n=15-16/prenatal treatment/sex for CUS). GD = gestational day; D = day; PN = postnatal day; OF = open field test; EPM = elevated plus maze; DL = dark-light emergence test; FST = forced swim test.

3.3.2 Behavior

Open field. In males, there were no effects of prenatal treatment or CUS on any open field measure (Figure 3.2**A,C,E,G,I**). By contrast, PAE and PF females, overall, showed fewer center entries than C females [main effect of treatment ($F_{2,63} = 3.766$, $p = 0.029$); Figure 3.2**B**]. As well, CUS decreased the number of center entries, time spent in the center, and distance travelled in the center regardless of whether testing occurred immediately (CUS-1) or following a delay (CUS-14) [main effects of CUS for center frequency: $F_{2,63} = 11.977$, $p < 0.001$; center duration: $F_{2,63} = 11.258$, $p < 0.001$; center distance: $F_{2,63} = 9.455$, $p < 0.001$; Figure 3.2**B,D,H**]. Additionally, latency to the first center entry was longer for CUS-1 than non-CUS animals overall [main effects of CUS ($F_{2,63} = 3.864$, $p = 0.026$); Figure 3.2**F**], and distance travelled in the entire field was lower overall for CUS-14 females than non-CUS females [main effect of CUS ($F_{2,63} = 5.583$, $p = 0.006$); Figure 3.2**J**].

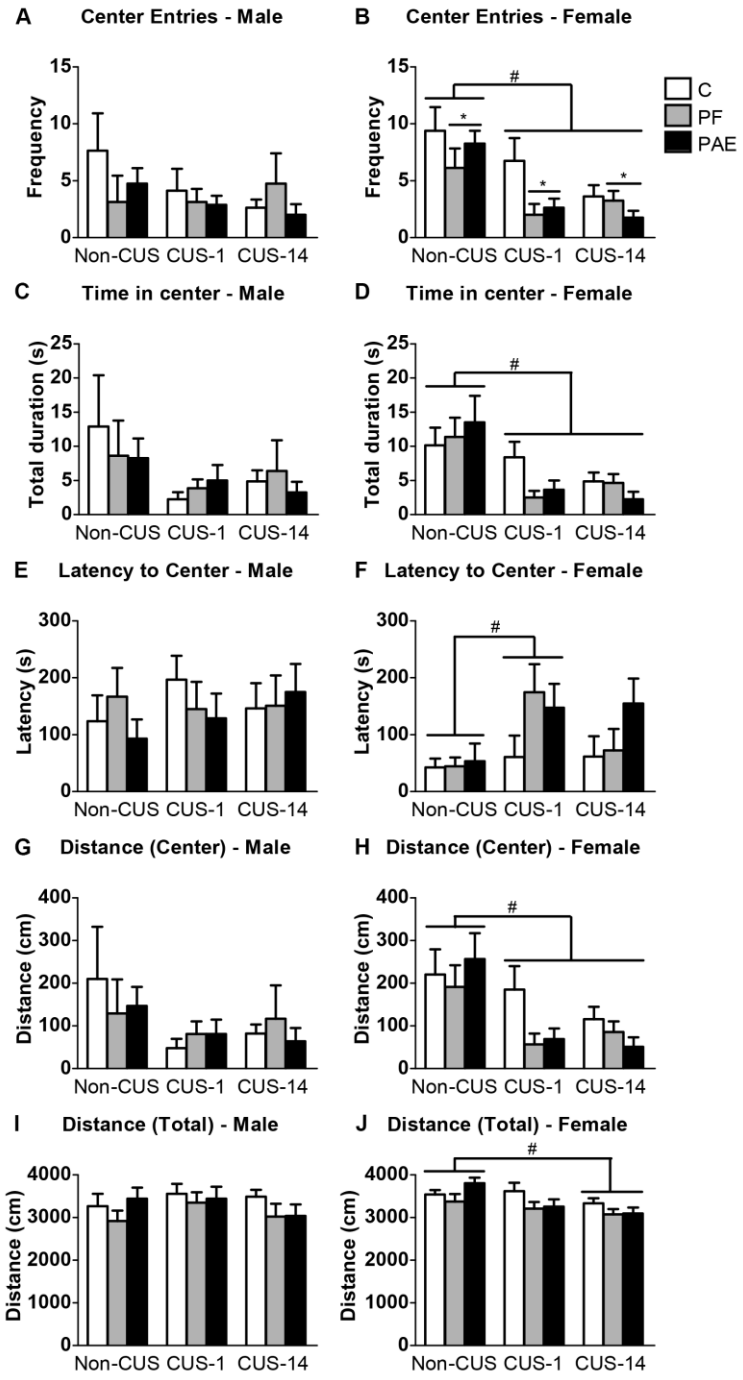

Figure 3.2 Immediate and delayed effects of chronic unpredictable stress (CUS-1 and CUS-14) and prenatal alcohol exposure (PAE) on behaviors in the open field.
Bars represent the mean ± SEM of frequency in center entries (**A,B**), time in the center (**C,D**), latency to center entry (**E,F**), distance travelled in the center (**G,H**), and total distance travelled in the field (**I,J**). * indicates a significant main effect of prenatal treatment: for **B**, PF and PAE are different from C; # indicates a significant main effect of CUS: CUS-1 and CUS-14 are different from non-CUS for **B,D,H**, CUS-1 is different from non-CUS for **F**, and CUS-14 is different from non-CUS for **J** (*n*=8/prenatal treatment/CUS condition/sex).

Dark-light emergence test. Latency to emerge was longer for PF than C males [main effect of treatment ($F_{2,60} = 3.376$, $p = 0.041$); Figure 3.3**A**]. Also, both PAE and PF males spent less time in the light than C males [main effect of treatment ($F_{2,60} = 3.255$, $p = 0.045$); Figure 3.3**C**]. Prenatal treatment had no effects in females (Figure 3.3**B,D**).

In addition, while ANOVA showed no effects of CUS in either males or females, *a priori* analyses revealed that CUS-14 PAE males showed longer latencies to emerge than CUS-14 C males ($F_{1,60} = 6.297$, $p = 0.015$).

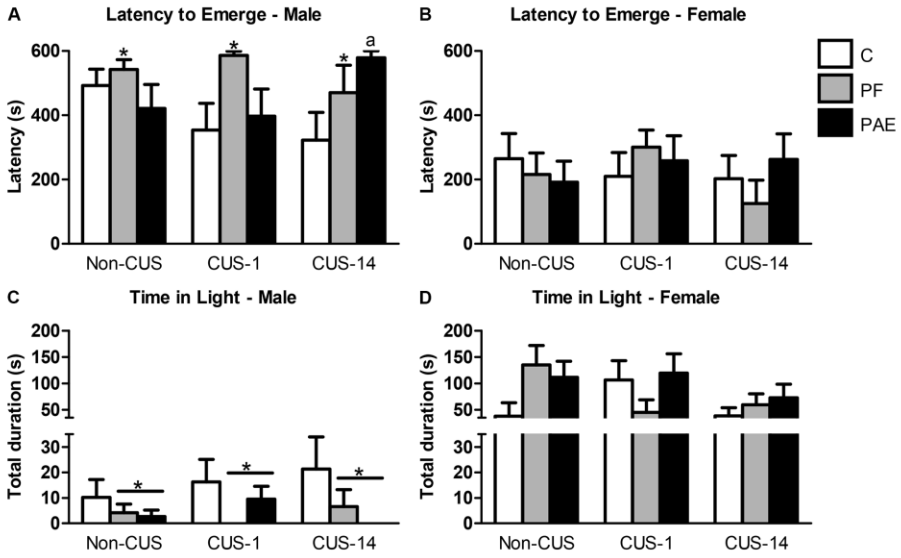

Figure 3.3 Immediate and delayed effects of chronic unpredictable stress (CUS-1 and CUS-14) and prenatal alcohol exposure (PAE) on behaviors in the dark-light emergence test.
Bars represent the mean ± SEM of latency to emerge from the dark (**A,B**) and time spent in the light compartment (**C,D**). * indicates a significant main effect of prenatal treatment: PF is different from C for **B**, and PF and PAE are different from C for **C**; [a] indicates that CUS-14 PAE is significantly different from CUS-14 C based on *a priori* comparisons (*n*=6-8/prenatal treatment/CUS condition/sex).

Elevated plus maze . There were no effects of prenatal treatment or CUS in either males or females on the percent of time in the open arms and the frequency of closed arm entries (data not shown).

3.3.3 Receptor mRNA expression

3.3.3.1 CRHR1 mRNA expression

Medial prefrontal cortex (mPFC). Neither prenatal treatment nor CUS altered CRHR1 mRNA expression in the mPFC or in the Cg1 and PrL, respectively, in either males or females. In the IL, however, CUS-1 females had higher CRHR1 mRNA expression than CUS-14 females [main effect of CUS ($F_{2,42} = 4.125$, $p = 0.023$); Figure 3.4**B**], while CRHR1 mRNA expression was decreased in PAE compared to C males in the CUS-1 condition (*a priori* analysis, $F_{1,41} = 5.846$, $p = 0.02$; Figure 3.4**A**).

Amygdala. Prenatal treatment had no effects in either males or females on CRHR1 mRNA expression in the amygdala (Figure 3.4). By contrast, CUS altered CRHR1 mRNA expression in the lateral and medial amygdala nuclei of males but not females, such that CUS-1 males showed higher mRNA expression than both non-CUS and CUS-14 males [main effect of CUS for lateral: $F_{2,41} = 4.864$, $p = 0.013$; medial: $F_{2,41} = 4.596$, $p = 0.016$; Figure 3.4**C,D,G,H**].

Hippocampal formation. CRHR1 mRNA expression was lower in the CA1 in PF than C males [main effect of treatment ($F_{2,41} = 3.704$, $p = 0.033$); Figure 3.4**I**]. There were no effects of prenatal treatment or CUS on CRHR1 mRNA expression in the hippocampal formation in females (Figure 3.4**J**).

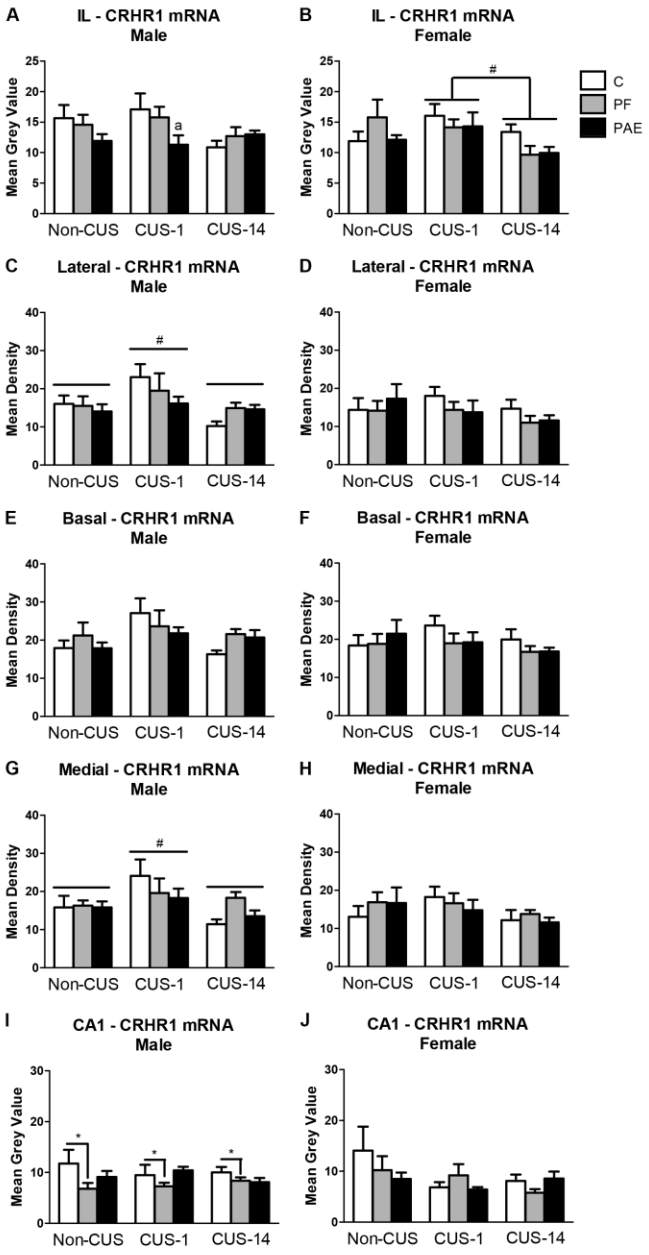

Figure 3.4 CRHR1 mRNA expression in the medial prefrontal cortex (mPFC), amgydala, and hippocampal formation in response to behavioral testing initiated 1- or 14-day following chronic unpredictable stress (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.

Bars represent mean grey values (mean ± SEM) of CRHR1 mRNA expression in the IL of the mPFC (**A,B**); lateral, basal, and medial nuclei of the amygdala (**C-H**); and CA1 subregion of the hippocampal formation (**I,J**). * indicates a significant main effect of prenatal treatment: PF is different from C for **I**; # indicates a significant main effect of CUS: CUS-1 is different from non-CUS and CUS-14 for **C,G**, and different from CUS-14 for **B**; [a] indicates that CUS-1 PAE is different from CUS-1 C based on *a priori* comparisons for **A** (*n*=4-6/prenatal treatment/CUS condition/sex).

3.3.3.2 MR mRNA expression

There were no effects of prenatal treatment in either males or females on MR mRNA expression in the hippocampal formation (Figure 3.5). Further, while there were no effects of CUS in males, CUS-14 females had lower mRNA expression overall than non-CUS females in the DG [main effect of CUS ($F_{2,44}$ = 3.543, p = 0.037); Figure 3.5**B**]. In the CA3, *a priori* analyses revealed that CUS-14 PAE females had higher mRNA expression than CUS-14 C females ($F_{1,44}$ = 4.567, p = 0.038; Figure 3.5**D**).

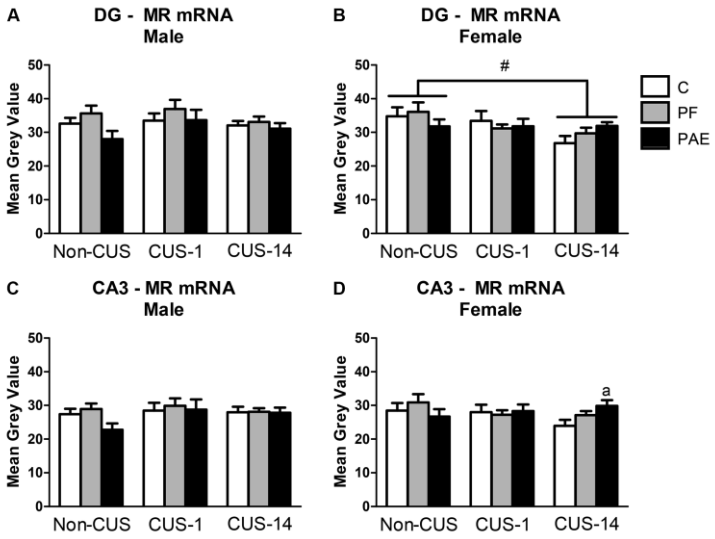

Figure 3.5 MR mRNA in the dentate gyrus (DG) and CA3 of the hippocampal formation in response to behavioral testing initiated 1- or 14-day following chronic unpredictable stress (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats. Bars represent mean grey values (mean ± SEM) of MR mRNA in the DG (**A,B**) and CA3 (**C,D**) subregions of the hippocampal formation. [#] indicates a significant main effect of CUS: CUS-14 is different from non-CUS; [a] indicates that CUS-14 PAE is different from CUS-14 C based on *a priori* comparisons (*n*=5-6/prenatal treatment/CUS condition/sex).

3.3.3.3 GR mRNA expression

Medial prefrontal cortex. There were no effects of prenatal treatment or CUS in either males or females on GR mRNA expression in the Cg1 or PrL subregions of the mPFC (data not shown).

However, in the IL, both PAE and PF females had decreased GR mRNA expression compared to C females [main effect of treatment ($F_{2,40} = 3.389$, $p = 0.044$); Figure 3.6**B**]. Furthermore, CUS altered GR mRNA expression in males but not females such that CUS-1 males showed higher GR mRNA expression than CUS-14 males [main effect of CUS ($F_{2,42} = 3.341$, $p = 0.045$); Figure 3.6**A**].

Amygdala. PAE males overall had lower GR mRNA expression than C and PF males in the medial nucleus of the amygdala [main effect of treatment ($F_{2,42} = 3.903$, $p = 0.028$); Figure 3.6**I**]. ANOVA also found that independent of prenatal treatment, CUS-1 males had higher GR mRNA expression than CUS-14 males in all nuclei of the amygdala [main effect of CUS for lateral: $F_{2,42} = 6.726$, $p = 0.003$; basal: $F_{2,42} = 4.703$, $p = 0.014$; central: $F_{2,42} = 3.434$, $p = 0.042$; medial: $F_{2,42} = 3.479$, $p = 0.40$; Figure 3.6**C,E,G,I**]. However, *a priori* analysis revealed that PAE males in the CUS-1 condition had lower GR mRNA expression than CUS-1 C males in the lateral ($F_{1,42} = 8.119$, $p = 0.007$), basal ($F_{1,42} = 7.758$, $p = 0.008$), and central ($F_{1,42} = 7.539$, $p = 0.009$) nuclei (Figure 3.6**C,E,G**).

By contrast, there were no prenatal treatment effects in females, and CUS-14 females showed lower mRNA expression compared to CUS-1 and non-CUS females in the lateral and basal nuclei, and compared to non-CUS females in the central nucleus [main effect of CUS for lateral: $F_{2,41} = 4.415$, $p = 0.018$; basal: $F_{2,41} = 4.184$, $p = 0.022$; central: $F_{2,41} = 3.382$, $p = 0.044$; Figure 3.6**D,F,H**].

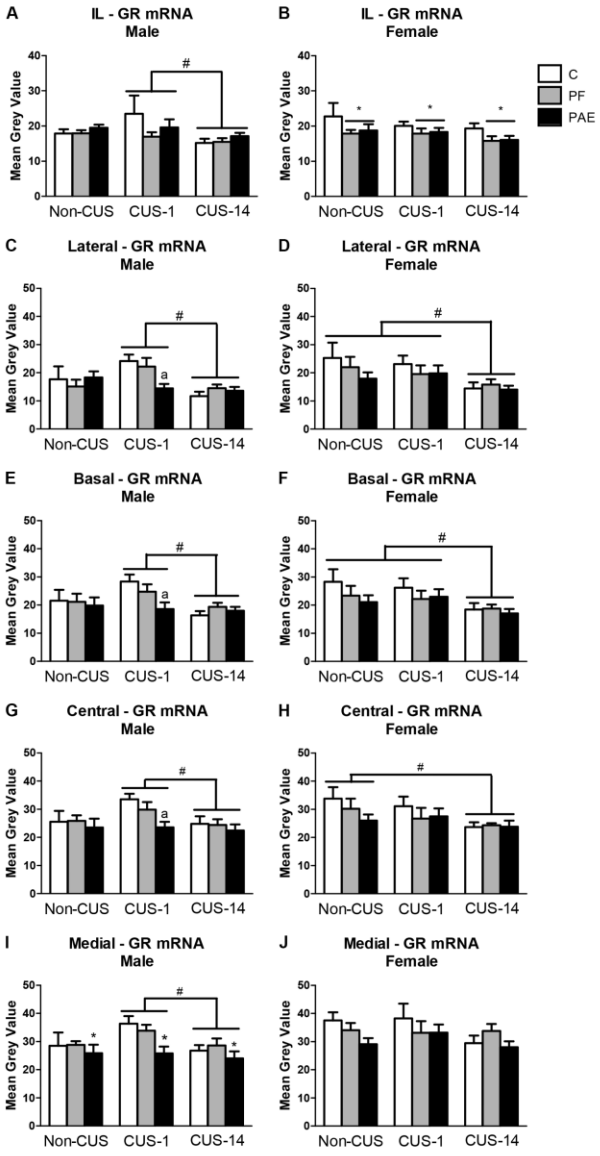

Figure 3.6 GR mRNA expression in the medial prefrontal cortex (mPFC) and amygdala in response to behavioral testing initiated 1- or 14-day following chronic unpredictable stress (CUS-1 and CUS-14, respectively) in adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats. Bars represent mean grey values (mean ± SEM) of GR mRNA expression in the IL of the mPFC (**A,B**); and lateral, basal, medial, and central nuclei of the amygdala (**C-J**). * indicates a significant main effect of prenatal treatment: PF and PAE are different from C for **B**, and PAE is different from both PF and C for **I**; # indicates a significant main effect of CUS: CUS-1 is different from CUS-14 for **A,C,E,G,I**, CUS-14 is different from both non-CUS and CUS-1 for **D,F**, and CUS-14 is different from non-CUS for **H**; [a] indicates that CUS-1 PAE is different from CUS-1 C based on *a priori* comparisons for **C,E,G** (*n*=4-6/prenatal treatment/CUS condition/sex).

Hippocampal formation. There were no effects of prenatal treatment or CUS in either males or females on GR mRNA expression in the hippocampal formation (data not shown).

3.4 Discussion

PAE and CUS, independently and interactively, altered anxiety-like behavior and CRHR1, MR, and GR expression in a time- and sex-dependent manner. Specifically, PAE differentially increased anxiety-like behavior and decreased GR mRNA in males and females compared to their control counterparts. Furthermore, depending on the timing of testing, CUS unmasked alterations in anxiety-like behavior, and GR and CRHR1 mRNA expression in the mPFC and amygdala in PAE males, and MR mRNA expression in the hippocampal formation in PAE females compared to their C counterparts. That CUS differentially affected brain and behavioral outcome of PAE and C animals, and did so in a sex-dependent manner, has important implications for understanding the etiology of psychopathology in individuals prenatally exposed to alcohol.

3.4.1 PAE differentially increased anxiety-like behavior and decreased expression of GR mRNA in males and females independent of CUS

The behavioral tests used in this study assessed anxiety-like behavior associated with the unconditioned response of rats in situations where there is a conflict between exploring novel environments and avoiding open spaces (Griebel and Holmes, 2013). Consistent with findings from previous studies, we found that PAE increased anxiety-like behavior, but the effects were sex- and test-dependent (Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Rouzer et al., 2017; Varlinskaya and Mooney, 2014). Specifically, regardless of CUS condition, PAE males showed greater anxiety-like behavior (less time in the light) compared to C males in the dark-light emergence test, while PAE females exhibited greater anxiety-like behavior (fewer center entries with no differences in locomotor activity) compared to C females in the open field.

These findings support previous data indicating that PAE has lasting impacts and may increase anxiety-like behavior in adulthood (Famy et al., 1998; O'Connor and Paley, 2009; Pei et al., 2011). The neurobiology that underlies behavior in each of these tests is not fully understood (Griebel and Holmes, 2013). However, studies demonstrating that rodent strains exhibiting moderate to high degrees of anxiety-like behavior perform differently in a battery of anxiety tests (van der Staay et al., 2009; van Gaalen and Steckler, 2000), and that subtle differences are found in the effects of several classes of anxiolytics (e.g. 5-HT_{1A} receptor agonists, adrenergic agents) on behavior in the elevated plus maze and dark-light emergence test (Bourin, 2015) provide support for the suggestion that different aspects of anxiety-like behavior may be measured by each test, and that acquiring results from a battery of tests is better than relying on findings from a single test. Together, our results suggest that PAE may alter different aspects of anxiety in males and females.

While there were no overall effects of PAE, independent of CUS, on CRHR1 and MR mRNA expression in either sex, PAE differentially decreased GR mRNA expression in males and females. We found that in the medial amygdala, PAE males, but not females, showed lower GR expression than their C counterparts. By contrast, in the IL of the mPFC, PAE females, but not males, exhibited lower GR mRNA expression than their C counterparts. The role of GR in mediating HPA activity following stress varies by brain area. In the mPFC, GR activation is involved with glucocorticoid negative feedback, but in the amygdala, it provides feedforward regulation of the HPA axis (Herman et al., 2016; Myers et al., 2012). Therefore, although there was GR downregulation in both males and females, it is possible that PAE females may have deficits in negative feedback regulation while PAE males may have deficits in feedforward

regulation of HPA activity. These findings are consistent with previous data showing that PAE differentially affects HPA activity/regulation in males and females (Glavas et al., 2007; Hellemans et al., 2010a). Given that PAE males and females both showed increased anxiety-like behavior albeit in different tasks, a decrease in GR mRNA expression may, at least in part, contribute to this altered behavior, although different mechanisms may be involved in males and females.

3.4.2 Differential immediate vs. delayed effects of CUS on stress-related receptor expression and anxiety-like behavior in males and females

We examined both immediate and delayed effects of CUS on brain and behavior. This allowed us to gain insight into the changes that occur immediately following chronic stress, while basal HPA activity is likely increased, compared to changes during a recovery period, when HPA activity likely returns to basal levels. Overall, we found that CUS unmasked alterations in mRNA expression of CRHR1 and GR, but not MR, in the brain of PAE males following immediate testing post-CUS, whereas the effects of CUS on anxiety-like behavior were apparent only with delayed testing. By contrast, CUS had delayed effects on MR, but not CRHR1 and GR, mRNA expression in the brain of PAE females, and there were no specific effects of CUS on behavior. Moreover, there was an overall effect of CUS on brain and behavior outcomes of females, independent of prenatal treatment.

Specifically, we found that PAE males failed to show the typical upregulation of CRHR1 and GR mRNA expression shown by their control counterparts following immediate testing post-CUS. That is, CRHR1 mRNA expression in the lateral and medial nuclei of the amygdala was

increased following immediate testing compared to both delayed testing and the non-CUS condition. GR mRNA expression was also higher following immediate compared to delayed testing in the IL, and in all nuclei of the amygdala. However, analyses indicate that CUS-induced increases in CRHR1 and GR mRNA expression were generally driven by C and PF males, with PAE males showing comparatively lower CRHR1 and GR mRNA expression in these brain areas. As noted, CRHR1 and GR have different roles in modulating HPA activity and behavior depending on where they are expressed (Henckens et al., 2016; Herman et al., 2016; Myers et al., 2012). Taken together, our findings suggest that PAE males may have deficits in modulating CRHR1- and GR-mediated HPA feedback and feedforward regulation, and in receptor autoregulation following CUS. Importantly, these results are consistent with our suggestion of a deficit in feedforward regulation in PAE males. Furthermore, given that basal CORT levels post-CUS were elevated similarly among groups, lower GR mRNA expression may indicate that PAE and C males respond differently to the same CUS-induced increase in basal CORT levels. Alternatively, as high CORT levels may downregulate GR (Saenz del Burgo et al., 2013), lower expression of GR mRNA in the mPFC and amygdala of PAE males immediately following CUS may reflect exposure to elevated CORT levels over the 10-day CUS period. This suggestion is consistent with previous findings, which demonstrate that CORT and/or ACTH levels are typically higher in PAE compared to control animals in response to a variety of stressors than C animals (reviewed in (Hellemans et al., 2010a)).

Interestingly, in comparison to the brain, the effects of CUS on anxiety-like behavior in PAE males were apparent only with delayed testing. Specifically, PAE males showed increased anxiety-like behavior (longer latency to emerge) compared to C males when testing occurred

following a recovery period, but not when testing occurred immediately after CUS. It is possible that the immediate changes in the brain set the stage for subsequent effects on behavior. For instance, both phosphorylation of trk B (the receptor for BDNF) and ERK (downstream kinase suggested to mediate antidepressant efficacy) were found to be decreased 2 weeks or 1 month, but not 1 day, following chronic corticosterone exposure (Gourley and Taylor, 2009). BDNF plays an important role in neuronal growth and survival, and in synaptogenesis and plasticity, and GR has been suggested to mediate the corticosterone-induced decrease in BDNF expression (Chen et al., 2017). In turn, altered BDNF expression has been suggested to contribute to the development of stress-related disorders, such as anxiety (Arango-Lievano et al., 2015). Therefore, a deficit in autoregulation of GRs in PAE males in response to CUS may have important implications for neuronal morphology and function, and may contribute to the delayed increase in anxiety-like behavior in PAE compared to C males.

By contrast to males, there were interactive effects of PAE and CUS on MR, but not GR and CRHR1, mRNA expression in females. MR mRNA expression in the CA3 subregion of the hippocampal formation was higher in PAE than C females following delayed testing post-CUS, suggesting that PAE females failed to show the typical downregulation of MR mRNA shown by their control counterparts following CUS, and thus may have a deficit in modulating MR expression. MR is involved in regulating both basal and stress-induced HPA activity, setting the threshold of HPA reactivity to stress, and maintaining high neuronal excitability (Joëls and de Kloet, 2017; ter Heegde et al., 2015). Furthermore, transgenic and pharmacological studies indicate that MR may be crucial for promoting neurogenesis, maintaining neuronal integrity, and preventing GR-mediated apoptosis (ter Heegde et al., 2015). Therefore, although we did not

observe interactive effects of PAE and CUS on anxiety-like behavior in females, our finding that differential MR mRNA expression was unmasked in PAE compared to C females following a recovery period after CUS suggests that changes in the brain may continue to occur after stress exposure has ended, with implications for sensitivity and stress reactivity of the HPA axis, neurogenesis, and neuronal integrity in the long-term.

In addition, we found both immediate and delayed effects of CUS on receptor mRNA expression and anxiety-like behavior in females, independent of prenatal treatment. Expression of CRHR1 mRNA in the IL was higher following immediate compared to delayed testing post-CUS. By contrast, MR mRNA expression in the DG and GR mRNA expression in the amygdala were lower with delayed testing compared to both immediate testing and/or non-CUS condition. Anxiety-like behavior was increased overall (i.e. decreased frequency of center entries, time in center, latency to center, and distance travelled in center) following both immediate and delayed testing post-CUS. CRHR1 has been suggested to mediate the anxiogenic effects of CRH in the mPFC, as CRH infusion in this brain area, which expresses only CRHR1, promotes anxiety-like behavior (Jaferi and Bhatnagar, 2007). Therefore, increased CRHR1 expression may contribute, at least in part, to the increase in anxiety-like behavior following CUS among females. The delayed effects of CUS on MR and GR mRNA expression suggest that CUS may alter both basal and stress regulation of HPA activity, but that dysregulation may not be manifest immediately. Taken together, it appears that CUS-induced alterations in CRHR1, MR and GR mRNA expression may underlie the increase in anxiety-like behavior among females. Furthermore, our finding of increased anxiety-like behavior following immediate testing post-CUS is consistent with previous studies on the short-term effects of CUS (see (Willner, 2005)), and our findings

extend the literature by demonstrating that certain effects of CUS on behavior may be delayed. Moreover, that CUS may selectively increase anxiety-like behavior in females is consistent with clinical literature suggesting that anxiety may be almost twice as common in women than in men, possibly due to increased susceptibility to environmental stress (Altemus, 2006; Sandanger et al., 2004).

3.4.3 Pair-feeding

The PF group, which receives a reduced ration of a nutritionally optimal diet matched in amount to that of an alcohol-consuming partner (Weinberg, 1985, 1984), is used to control for the reduction in food intake typically observed in alcohol-consuming animals. However, pair-feeding is an imperfect control group as it cannot control for the nutritional effects of alcohol (e.g. absorption and utilization of nutrients). In addition, because they receive a reduced ration of food, these animals also tend to consume their entire daily ration within a few hours of diet presentation, and are thus food deprived and hungry until feeding the next day. Therefore, a component of prenatal stress is likely associated with the pair-feeding paradigm and effects on offspring represent, at least partially, the impact of combined effects of prenatal stress and the nutritional consequence of receiving a reduced food ration. Our behavioral data show that both PF males and females, regardless of CUS exposure, exhibited increased anxiety-like behavior compared to their respective C counterparts. Furthermore, we found that pair-feeding decreased CRHR1 mRNA expression in the CA1 in males and decreased GR mRNA expression in the IL in females. More importantly, however, while PF males and females may in a few cases show similar changes in brain and behavioral measures as PAE animals, in other cases they exhibit different mRNA expression and behavior. Taken together, these results indicate that while some

effects of PAE and PF may overlap, the mechanisms underlying PAE and PF effects likely differ rather than representing a continuum of effects along the same pathway (Glavas et al., 2007).

3.4.4 Limitations

It is possible that mechanisms beyond those investigated in the present study might play a role in the functional effects of PAE that were observed. For example, while there were several instances where there were no effects of PAE on receptor mRNA expression, changes in receptor localization could also be an issue in the altered anxiety-like behavior observed. Indeed, it has been reported that while total levels of GR and MR were unchanged in unstressed adolescent PAE mice, nuclear localization of the receptors in the hippocampus and PFC was altered, and these impairments were associated with decreased hippocampus-dependent learning and memory, and inflexibility in frontal cortical-dependent reversal learning (Allan et al., 2014; Caldwell et al., 2014). Additionally, membrane-bound GR and MR are known to exert non-genomic effects distinct from those of cytoplasmic/nuclear receptors, in response to stress (Joëls and de Kloet, 2017; Rainville et al., 2017). Further investigations using an integrated approach in examining the complex molecular machinery of MR- and GR-mediated regulation of the HPA stress response following PAE will certainly enrich the current findings.

Another limitation in the present experimental design is that behavioral tests were conducted without counterbalancing for order of testing. The order of testing was found to have only a minor impact on behavioral responses, except when a more stressful behavioral test, such as FST, is done first or last (Blokland et al., 2012; McIlwain et al., 2001). Our typical practice is to order the tests from least to most stressful, e.g., FST is typically performed last. The possibility

that PAE animals may respond differently from controls to the cumulative stress from a particular testing order remains to be determined and could provide additional insight into the outcomes we observed. As it stands, the present study does provide important information on brain and behavioral responses to the same cumulative stress experience, which has implications for the response to stress in general in individuals prenatally exposed to alcohol. It would be an interesting extension to the current findings to probe for possible prenatal treatment x order of testing x CUS exposure interactions to further evaluate the impact of PAE on anxiety-like behavior, which could enhance interpretations of the present results.

3.5 Conclusions

In the present study, we found that PAE and CUS independently increased anxiety-like behavior and altered MR, GR, and CRHR1 mRNA expression, and did so in a sexually dimorphic manner. In addition, exposure to CUS differentially unmasked alterations in anxiety-like behavior and receptor mRNA expression in the mPFC, amygdala, and hippocampal formation in PAE males and females. These results substantiate sex differences in vulnerability to stress and anxiety, and suggest that different mechanisms may underlie anxiety-like behavior in males and females. That the effects of CUS can be immediate or delayed underscores the importance of investigating the temporal effects of CUS for a more thorough understanding of the role of HPA activity in anxiety-like behavior. Overall, the changes observed in the receptor systems in brain areas involved with both stress and emotional regulation could potentially underlie the increase in anxiety-like behavior following PAE and CUS exposure in adulthood. Delineating the mechanisms by which PAE and CUS, independently and interactively, may contribute to the

development of mental health issues, such as anxiety, will ultimately help establish novel or targeted interventions and treatments for affected individuals.

Chapter 4: Role of corticosterone in anxiety-like behavior and HPA regulation following prenatal alcohol exposure: Sex differences in outcome

4.1 Introduction

Prenatal alcohol exposure (PAE) is known to cause dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, including hyperresponsivity to stressors. For example, higher basal and stress cortisol levels have been reported in infants and toddlers following PAE (Haley et al., 2006; Jacobson et al., 1999; Ramsay et al., 1996), and studies using animals have demonstrated, in a sex-dependent manner, increases in HPA activation and/or a delayed return to basal corticosterone levels in PAE compared to control offspring in response to a wide range of stressors (Lee et al., 2000, 1990; Lee and Rivier, 1996; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1988; Weinberg et al., 2008). Furthermore, individuals prenatally exposed to alcohol are at a higher risk than unexposed individuals of encountering stressful environments during their lifetimes (O'Connor and Paley, 2006; Streissguth et al., 2004, 1991), which could exacerbate HPA dysregulation/hyperresponsivity. Dysregulation of the HPA axis is known to play a role in vulnerability to stress-related disorders, such as depression and anxiety (Jacobson, 2014; Nestler et al., 2002). Therefore, it is possible that PAE-induced HPA dysregulation or activity could predispose individuals to an increased vulnerability to stress-related disorders following exposure to stressors over the life course.

Indeed, individuals prenatally exposed to alcohol have an increased risk of developing one or more mental health problems later in life, with depression and anxiety being among the most commonly encountered disorders (Famy et al., 1998; O'Connor et al., 2002; O'Connor and Paley, 2009; Pei et al., 2011; Streissguth et al., 2004, 1991). Preclinical studies support the

clinical findings that PAE can increase anxiety- and depressive-like behavior (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Rouzer et al., 2017; Varlinskaya and Mooney, 2014; Wilcoxon et al., 2005). Importantly, chronic unpredictable stress (CUS) differentially exacerbates anxiety- and depressive-like behavior in PAE and control animals, and effects are sex dependent (Hellemans et al., 2010a, 2010b; Lam et al., 2018b [**Chapter 2**]; Raineki et al., 2016). However, it is unclear whether increased secretion of corticosterone in response to stress *per se* is involved with mediating differential effects of CUS in PAE and C animals.

Two key receptors are involved in regulating HPA activity by mediating the effects of the glucocorticoids: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). MRs are implicated in regulating basal HPA tone, and may also play a role in setting the threshold of HPA reactivity to stress and maintaining high neuronal excitability (Joëls and de Kloet, 2017; ter Heegde et al., 2015). On the other hand, GRs appear to be involved primarily in mediating feedforward/feedback regulation of the stress response (Herman et al., 2016). Importantly, dysregulation of MR and GR has been suggested to play a role in the psychopathology underlying depression and anxiety (Binder and Nemeroff, 2010; Holsboer and Ising, 2010, 2008; Inda et al., 2017; Joëls and de Kloet, 2017; Veenit et al., 2014). PAE can alter, in a sex-dependent manner, MR and GR mRNA expression. PAE decreases MR mRNA expression in the adult female hippocampus (Sliwowska et al., 2008; Uban et al., 2013), and alters hippocampal MR mRNA expression in adolescent male and GR mRNA expression in adolescent female rats (Raineki et al., 2018). Furthermore, PAE differentially decreases medial prefrontal cortex (mPFC) and amygdala GR mRNA expression in adult males and females (Lam et al., 2018a

[**Chapter 3**]). As such, dysregulation of these key receptor systems by PAE may predispose individuals to the adverse effects of stress over the life course – which may in turn exacerbate HPA dysregulation – and increase vulnerability to stress-related disorders.

PAE also alters the role of corticosterone in regulating expression of these receptors. Removal of endogenous corticosterone feedback by adrenalectomy (ADX) revealed higher hippocampal GR and MR mRNA expression in PAE males and females, respectively, than their C counterparts, and while corticosterone replacement at basal physiological levels normalized these PAE effects following ADX, it is insufficient in PAE compared to C males at normalizing the ADX-induced increase in hippocampal MR mRNA expression (Glavas et al., 2007). However, the role of corticosterone in regulating emotional behavior in the context of PAE has not been assessed.

The present study builds on and extends previous findings to determine whether PAE-induced HPA hyperresponsivity to stress, corticosterone hypersecretion in particular, mediates the differential effects of CUS on behavior and expression of MR and GR mRNA in PAE and C animals. We first identified the effects of PAE and CUS on behavior, and MR and GR mRNA expression in adrenal-intact animals that underwent sham surgery. Animals were exposed to CUS and anxiety-like behaviors were evaluated using the open field and elevated plus maze tests. Expression of MR and GR mRNA was assessed in the mPFC, amygdala, and hippocampal formation, brain areas key to both stress and emotional regulation. Then we utilized ADX with corticosterone replacement (ADXR) at basal physiological levels to normalize corticosterone levels in adult PAE males and females, and examined whether effects of PAE and/or CUS on behavior, and MR and GR mRNA expression were attenuated or reversed.

4.2 Methods

4.2.1 Animals and breeding

All animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, the Canadian Council on Animal Care, and approved by the University of British Columbia Animal Care Committee. Adult virgin male (275-300g) and female (265-300g) Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, PQ, Canada). Rats were pair-housed by sex on corn cob bedding, and habituated to the Centre for Disease Modeling which was maintained on a 12:12 hour light/dark cycle (lights on at 07:00 h) with controlled temperature (21±1°C) for a 7-10-day period. During habituation, animals had *ad libitum* access to 18% protein chow (Teklad Global #2018) and water. For breeding, males were singly housed, and a female and male were paired. Presence of sperm in vaginal lavage samples taken every morning at 08:00 h indicated day 1 of gestation (GD 1).

4.2.2 Diets and feeding

On GD 1, females were singly housed in polycarbonate cages on ventilated racks with corn cob bedding. Dams were randomly assigned to one of three treatment groups: 1) alcohol-fed (PAE; n=13), receiving a liquid ethanol diet with 36% ethanol-derived calories, 6.7% v/v; 2) pair-fed (PF; n=11), receiving a liquid control diet with maltose-dextrin isocalorically substituted for ethanol, in an amount consumed by a PAE partner (g/kg body weight/day of gestation); and 3) *ad libitum*-fed control (C; n=10), receiving a pelleted control diet. All diets were formulated to provide optimal nutrition during pregnancy (Weinberg/Keiver High Protein Ethanol [#710324]

and Control [#710109] diets; as well as Weinberg/Keiver High Protein Pelleted Control Diet [#710109] from Dyets, Inc., Bethlehem, PA, USA) (Lan et al., 2006).

The diets were presented fresh daily, one hour prior to lights off to minimize shifts in the maternal corticosterone circadian rhythm (Gallo and Weinberg, 1981; Krieger, 1974), and at that time the volume of liquid diets consumed since the previous night was recorded. All groups also received *ad libitum* access to water. Liquid ethanol diets were introduced gradually over the first 2 days of pregnancy with a 1:2 ratio of liquid ethanol to liquid control diet on GD 1 and 2:1 ratio on GD 2 to facilitate the transition into a full liquid ethanol diet beginning on GD 3. On GD 17, blood alcohol levels, from blood samples collected 3-hr after lights off from the tail vein, were determined using an assay from Pointe Scientific Inc. (Canton, MI, USA) to be 122.0 ± 10.1 mg/dl in PAE dams. Experimental diets continued through GD 21. Beginning on GD 22 and throughout lactation, ethanol-fed dams were switched to a 19% high protein laboratory chow (Teklad Global #2019) and water to minimize adverse effects of ethanol on maternal lactation. Dams from the other two diet groups were also given a 19% high protein laboratory chow (Teklad Global #2019) beginning GD22. At birth, pups were weighed, and litters randomly culled to 12 (6 males, 6 females when possible). If necessary, pups from the same prenatal group born on the same day were fostered into a litter to maintain the litter size. Dams and offspring were weighed weekly but were otherwise undisturbed until weaning on postnatal day (PN) 22 (see Appendix A for developmental data), after which pups were group housed by litter and sex until they were pair-housed at PN 40±2. Animals of the same sex and from the same prenatal group, but from different litters born ±2 days were paired. Weaned pups and adults were fed an 18% protein chow (Teklad Global #2018) and housed on non-ventilated racks.

4.2.3 Adrenalectomy and corticosterone replacement (ADXR)

A pilot experiment was first done in adult (PN 55-65) control males and females (n=10 per sex) that were not part of the present study to determine the effectiveness of the ADXR procedure in simulating typical morning (trough; 09:00h) and evening (peak; 22:00h) basal corticosterone levels. Bilateral ADX was carried out via a dorsal approach under isoflurane anesthesia; sham surgery involved the same procedures except the adrenal glands were not removed. Immediately following ADX, corticosterone was provided in the drinking water (0.9% NaCl, 0.2% ethanol), at concentrations of 25 and 75 μg⁄ml for males and females, respectively, and animals were followed for 21 days, with blood sampling on days 5, 14, and 21 for determination of plasma corticosterone levels.

ADXR procedures in the present study were identical to those in the pilot experiment above, except blood sampling was not done on days 5, 14 and 21 to avoid additional stress. In adulthood (PN 55-65; **FIGURE 4.1**), males and females from each prenatal group (C, PF, and PAE) were randomly assigned to either the Sham or ADXR surgical condition. Only one male and one female offspring from each litter were assigned to any one condition. Corticosterone was provided in drinking water immediately following ADX in concentrations as described above to clamp plasma corticosterone to low basal levels. Twenty-four-hr corticosterone intakes pre-CUS (the day before and Day 1 of CUS) and post-CUS (Day 10, and the day after CUS ended) were recorded in ADXR animals. The corticosterone concentration for males was chosen based on previous studies, which demonstrated that this level of corticosterone replacement results in basal plasma corticosterone levels, and normalizes basal ACTH levels and thymus weight compared with ADX alone (Akana et al., 1985; Glavas, 2003; Glavas et al., 2001; Jacobson et

al., 1988). Replacement levels for females were determined through previous pilot testing and were shown to normalize thymus weight (Glavas, 2003).

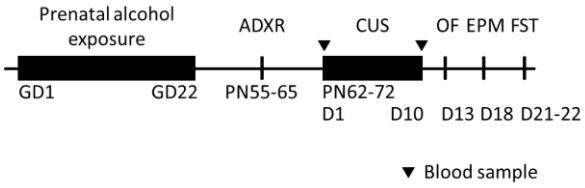

Figure 4.1 Experimental design.
ADXR = adrenalectomy with corticosterone replacement; D = day; EPM = elevated plus maze; FST = forced swim test; GD = gestational day; OF = open field test; PN = postnatal day.

4.2.4 Chronic unpredictable stress (CUS) paradigm

Following a 6-day rest from surgery, animals from each experimental group were randomly assigned to either stressed (CUS) or non-stressed (non-CUS) conditions. Only one male and one female offspring from each litter were assigned to any one condition. CUS involved 10 days (**FIGURE 4.1**) of twice daily unpredictable exposure to mild stressors, with a minimum of 2 hr between stressors. All CUS rats were exposed, in variable order, to the same number of each stressor over the 10-day period.

Stressors used: Platform: Animals placed on 20 x 20 cm transparent Plexiglas platforms, mounted on 90 cm high posts, for 20 min. Restraint: Animals restrained in PVC tubes (15 cm × 6 cm for females and 19 cm × 7 cm for males) with ventilation holes for 30 min. Soiled Cage: Animals placed in a cage with soiled bedding from other same-sex animals for 1 hr. Social isolation: 12 hr of isolation beginning at lights off with food and either water (Sham) or water with corticosterone (ADXR animals). Animals are returned to the home cage in the morning.

Cage Tilt: Home cages tilted to a 30° angle for 2 hr. Novel Cage: Animals housed in pairs in a new cage without bedding, food, or water for 1 hr. Non-CUS animals were left undisturbed except for regular cage changes.

4.2.5 Behavioral testing

Behavioral testing began the third day after the end of CUS (**FIGURE 4.1**). All animals were assessed on consecutive days, with a one-day break between tests, in the open field, elevated plus maze, and forced swim tests. The current paper focuses on the results from the open field and elevated plus maze to examine the relationship between anxiety-like behavior and MR and GR mRNA expression. 24 hr prior to behavioral testing, animals were habituated to the testing room for 20 min. n = 9-11 per prenatal treatment/CUS exposure/surgical condition/sex. Description of the tests are as follows:

The open field apparatus was a square arena (80 x 80 cm) enclosed by transparent Plexiglas walls (40 cm). Animals were tested for 5 min in the field under bright lighting. Total distance traveled (cm) in the field, as well as distance travelled, time spent, and entries into the center zone were analyzed. Data for the open field were recorded and automatically analyzed using the MotorMonitor™ software.

The elevated plus maze was elevated 40 cm above ground and consisted of two open arms and two arms enclosed by 40 cm opaque walls (each arm is 50 x 10 cm, and the center area is 10 x 10 cm). Under dim lighting, animals were individually placed on the center of the maze facing an open arm and were allowed to roam freely for 5 min. Time on the open arms as a percent of open

and closed arms time, and frequency of closed arm entries were assessed. Entry into an arm is defined as when all four paws are in the zone. Elevated plus maze behaviors were recorded and scored using The Observer 5.0 software (Noldus, Wageningen, The Netherlands).

All testing was done during the light phase of the circadian cycle. White noise was played at 30 dB in the background during testing to dampen random noise. All behaviors were analyzed by an observer blind to the prenatal group and stress exposure.

4.2.6 Blood sampling and tissue collection

Basal blood samples at the circadian trough were collected via tail nick from both CUS and non-CUS animals on Day 1 of CUS and on the day after CUS ended at 1 hr after lights on. Samples were collected within 2 min of touching the cage to obtain a true basal measure. Blood was collected on ice in plastic centrifuge (tail nick) containing 5µl/100 µl blood of EDTA (anti-coagulant; Fisher Scientific, ON, Canada) and 2.64 µg/100 µl blood of aprotinin (anti-protease; Sigma-Aldrich, ON, Canada). Blood was centrifuged within 60 min of sample collection at 3200rpm for 10 min at 4°C, and the plasma was stored at -20°C until assayed.

Whole brains were collected via decapitation 30 min after testing on Day 2 of the forced swim test. Brains were collected and snap frozen on powdered dry ice and stored at -80°C.

4.2.7 Radioimmunoassays (RIAs)

Corticosterone: Total corticosterone levels (bound plus free) were measured using a modification of the ImmuChem^{TM} Corticosterone I^{125} RIA Kit (MP Biomedicals, Orangeburg,

NY); all reagents and samples were halved, and assay was performed according to the vendor instructions. The minimum detectable range was 7.7 ng/ml, and the inter- and intra-assay coefficients of variation were <7.2% and <10.3%, respectively, as provided by the vendor.

ACTH: The ImmuChem^{TM} Double Antibody hACTH I^{125} RIA Kit (MP Biomedicals, Orangeburg, NY) was used. The minimum detectable range was 5.7 pg/ml, and the inter- and intra-assay coefficients of variation were <10.7% and <6.8%, respectively, as provided by the vendor.

4.2.8 *In situ* hybridization

20μm coronal sections were collected on a cryostat at -16 ^{o}C, mounted on slides (Superfrost slides, Fisher Scientific, ON, Canada), and stored at –80^{o}C. For all brain areas, n = 6-7 per prenatal treatment/CUS exposure/surgical condition/sex.

Probe and labeling: Ribonucleotide probes were used to detect GR mRNA in the medial prefrontal cortex (mPFC; prelimbic [PrL] and infralimbic [IL] cortices), amygdala (central, medial, lateral, and basal nuclei), and the hippocampal formation (dentate gyrus [DG], CA3, CA1, and ventral subiculum). The rat GR ribonucleotide probe was prepared using a 456 bp template (complementary to the coding region and 3' untranslated region of rat GR mRNA) and was provided by Dr. James Herman (Department of Psychiatry and Behavioral Neuroscience, College of Medicine, University of Cincinnati, USA) (Herman et al., 1999). A ribonucleotide probe was also used to detect MR mRNA in the hippocampal formation, and it was prepared using a 550 bp template (complementary to the coding region and 3' untranslated region of rat

MR mRNA) also from Dr. James Herman (Herman et al., 1999). The ribonucleotide probes were labeled with 35S-UTP (Amersham Biosciences, NJ, USA) using Polymerase T7 (GR) or T3 (MR) and Promega Riboprobe System (Promega Corporation, Madison, WI, USA). All probes were purified using Micro Bio-Spin 30 Columns (Bio-Rad, CA, USA). 1M of DTT was added to prevent oxidation.

Hybridization: The slides were thawed for 20 min, fixed in formalin for 30 min, and then pre-hybridized as follows: 1x PBS twice for 10 min each, proteinase K (100 μg/L) digestion at 37ºC for 9 min, 0.1M triethanolamine-hydrochloride (TEA) for 10 min, acetylation by 0.1M TEA with 0.25% acetic anhydride for 10 min, 2x sodium saline citrate (SSC) twice for 5 min each, dehydration through a graded series of ethanol, delipidation in chloroform for 5 min, and finally 100% ethanol before being air-dried. Hybridization buffer (75% formamide, 3X SSC, 1X Denhardt's solution, 200 μg/mL yeast tRNA, 50 nM sodium phosphate buffer (pH 7.4), 10% dextran sulphate, and 10mM DTT) was applied (1 x 10^6 cpm/slide) and covered with HybriSlips (Sigma-Aldrich, ON, Canada). The sections were incubated overnight at 55ºC in humidified chambers (75% formamide). HybriSlips were then removed and slides were rinsed as follows: 2x SSC twice for 20 min, 2x SSC for 30 min, 50 μg/L RNAse A solution at 37 ºC for 60 min, 2X SSC with 0.01M DTT for 10 min, 1X SSC for 10 min, 0.5X SSC with 0.01M DTT for 10 min, 0.1X SSC with 0.01M DDT at 60 ºC for 60 min, and 0.1X SSC for 15 min. The sections were then dehydrated with a graded series of ethanol, and finally air dried overnight.

Kodak BioMax MR autoradiography film was exposed to hybridized slides of the medial prefrontal cortex (mPFC), amygdala (GR), and hippocampal formation. Exposure time were as

follows: 14 days for GR in the mPFC; 26 days for GR in the amygdala; and 8 days for GR and 7 days for MR in the hippocampal formation. The exposed autoradiography films were developed using Kodak GBX developer and fixer.

Densitometric analysis: The autoradiographic films were scanned and analyzed using Scion Image 4.0.3.2 (National Institutes of Health, USA) according to the Paxinos and Watson Stereotaxic Rat Brain Atlas, Fifth edition (Paxinos and Watson, 2004). Mean grey density levels were measured from Bregma 3.00 mm to 2.76 mm for the mPFC; Bregma -2.64 mm to -3.00 mm for the amygdala; and Bregma -4.80 mm to -5.28 mm for the hippocampal formation. Consistent with other studies in the literature, the Bregma range chosen for the hippocampal formation includes the ventral/temporal hippocampus which primarily relates to emotion and stress regulation (Abela et al., 2013; Bast et al., 2009; Burton et al., 2009; Consolo et al., 1994; Fanselow and Dong, 2010; Ferbinteanu and McDonald, 2000; Strange et al., 2014). The left and right sides of each brain subregion were traced freehand in two sections per animal for a total of four measurements to determine mean gray density levels. Background was measured from the forceps minor for mPFC, the internal capsule for the amygdala, and corpus callosum for the hippocampal formation. Corrected gray levels were obtained by subtracting the background level from each of the four measurements and the four measurements were then averaged together for analysis.

4.2.9 Statistical Analyses

Extreme outliers lower than 3 interquartile ranges below the first quartile or higher than 3 interquartile ranges above the third quartile were identified and removed prior to statistical

analyses (see Appendix C for details). Due to known sex differences of HPA regulation and that main effects of sex were revealed for corticosterone results and several behavioral measures (see Appendix D), all data were analyzed separately for males and females. AM and PM basal corticosterone data from control animals from the pilot study were compared using a paired t test using IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 software (IBM, Armonk, NY, USA). Pre-CUS corticosterone intake (mg per kg body weight) of ADXR animals was analyzed by one-way analysis of variance (ANOVA) for the factor of prenatal treatment (C/PF/PAE). Pre-CUS basal corticosterone data were analyzed by two-way ANOVA for the factors of prenatal treatment and surgical condition. Post-CUS corticosterone intake (mg per kg body weight) of ADXR animals was analyzed by two-way ANOVA for the factors of prenatal treatment and CUS exposure. All other data were analyzed by three-way ANOVA for the factors of prenatal treatment, surgical condition, and CUS exposure. All ANOVAs were followed by Šídák *post hoc* correction to examine significant main effects and interactions. Differences were considered significant at $p \leq 0.05$. Further analyses on the behavioral and brain data utilized planned comparisons to test the *a priori* hypotheses that: 1) PAE will alter depressive- and anxiety-like behavior and mRNA levels of MR and GR in brain areas involved with both HPA and emotional regulation; 2) CUS will differentially alter behavior and mRNA levels of MR and GR in PAE compared to control animals; and 3) clamping basal corticosterone levels by ADXR will attenuate or normalize the effects of PAE and CUS on behavior and MR and GR mRNA levels. *A priori* hypotheses 1 and 2 refer to animals in the sham condition, and planned comparisons were made between PAE and C animals within the same CUS condition (e.g. non-CUS PAE vs non-CUS C). *A priori* hypothesis 3 refers to animals in the ADXR condition, and the same planned comparisons used to address hypotheses 1 and 2 were performed. P values

from ANOVAs and *a priori* analyses are cited in the Results section; p values from *post hoc* and *a priori* comparisons are indicated in the Figures and noted in the Figure Legends.

4.3 Results

4.3.1 Pilot Study: AM and PM basal corticosterone levels in ADXR control animals

Morning and evening corticosterone levels were significantly different from each other in both female [$t(9) = -7.037$, $p < 0.001$] and male [$t(9) = -9.521$, $p < 0.001$] controls following ADXR (Figure 4.2). Importantly, replacing corticosterone by putting it in the drinking water of ADX animals resulted in a strong circadian rhythm and relatively low variability in corticosterone levels achieved.

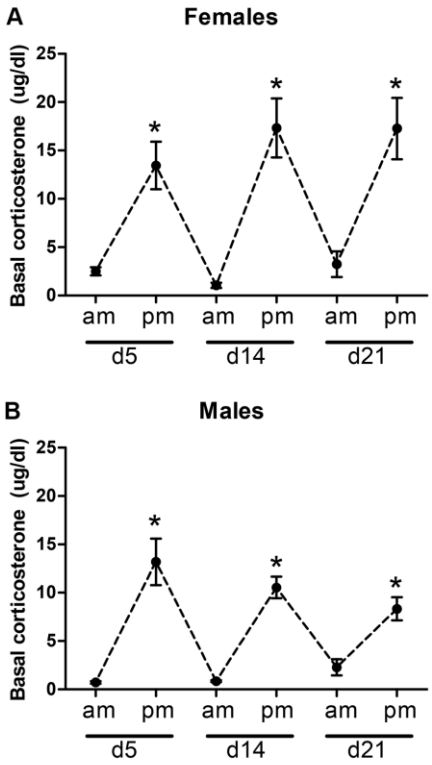

Figure 4.2 AM and PM basal corticosterone levels in control animals following adrenalectomy with corticosterone replacement.
PM basal corticosterone levels (mean ± SEM) were significantly higher than AM levels in both males and females (* $p<0.05$). *n*=10 per sex.

4.3.2 Corticosterone intake (mg/kg body weight) and changes in body weight

There were no effects of prenatal treatment on pre-CUS corticosterone intake, and no effects of prenatal treatment or CUS exposure on post-CUS corticosterone intake in ADXR animals (data shown in Appendix B).

There were no significant effects of prenatal treatment, surgical condition, or CUS exposure on changes in body weight in females. By contrast, in males, CUS decreased weight gain (main effect of CUS exposure: $F_{1,111} = 38.727$, $p < 0.001$). In addition, prenatal treatment interacted with surgical condition to alter body weight (prenatal treatment x surgical condition interaction:

$F_{2,111} = 4.208$, $p = 0.017$; Figure 4.3). In the ADXR condition, PAE gained less weight than C males, and both PAE and PF males gained less weight than their sham counterparts.

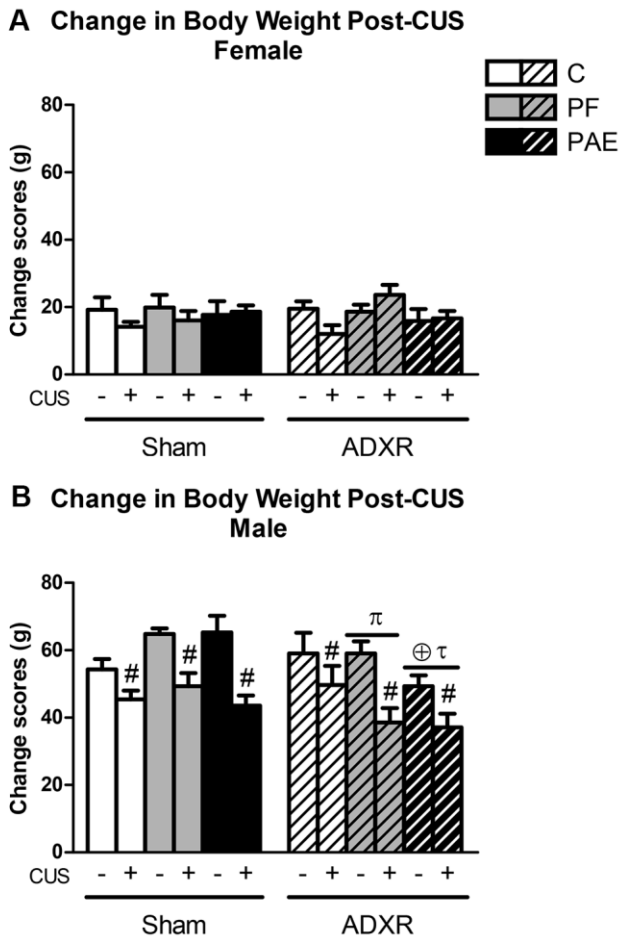

Figure 4.3 Change in body weight following adrenalectomy with corticosterone replacement (ADXR) and CUS exposure.

Bars represent change scores (difference between pre- and post-CUS body weight; mean ± SEM) following the 10-day period of CUS in control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) females (**A**) and males (**B**). For **B**, $^{\#}$ CUS males gained less weight less than non-CUS males ($p < 0.001$); within the ADXR condition, $\oplus$ PAE males gained less weight than their C counterparts ($p = 0.020$); and between surgical conditions, $^{\pi}$ PF ($p = 0.045$) and $^{\tau}$ PAE ($p = 0.007$) males under the ADXR condition gained less weight than their respective sham counterparts. In the x-axis, $^{-}$ indicates non-CUS while $^{+}$ indicates CUS animals. *n* = 10-11/prenatal treatment/surgical condition/CUS exposure/sex.

4.3.3 Basal corticosterone and ACTH levels

Pre-CUS corticosterone. Pre-CUS basal corticosterone levels were differentially altered in females and males, with lower levels in ADXR females and higher levels in ADXR males compared to their respective sham counterparts (main effect of surgical condition – females: $F_{1,108} = 11.838$, $p = 0.001$; male: $F_{1,109} = 4.313$, $p = 0.040$; Figure 4.4**A,C**).

Pre-CUS ACTH. In both females and males, pre-CUS basal ACTH levels were higher in the ADXR than the sham condition (main effect of surgical condition – females: $F_{1,106} = 27.482$, $p < 0.001$; males: $F_{1,112} = 19.145$, $p < 0.001$; Figure 4.4**B,D**).

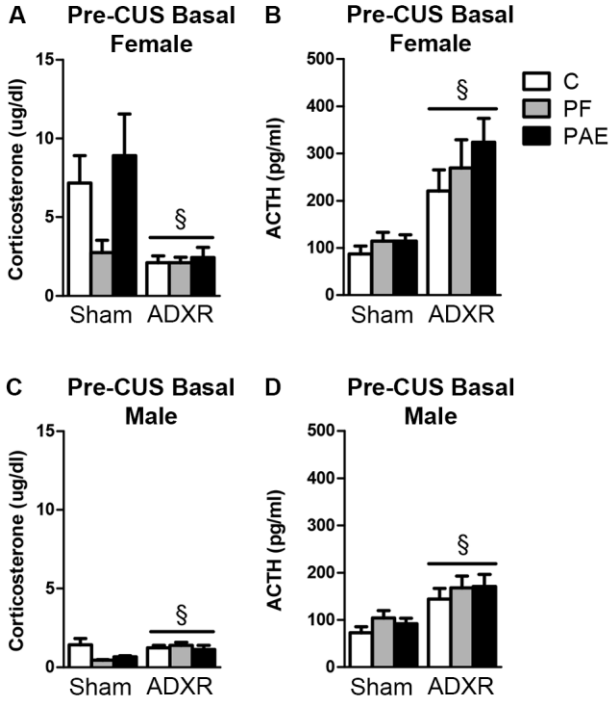

Figure 4.4 Effects of prenatal alcohol exposure (PAE) and adrenalectomy with corticosterone replacement (ADXR) on pre-CUS basal corticosterone (µg/dL) and ACTH (pg/ml) levels in females (A,B) and males (C,D). Bars represent mean ± SEM. § animals under the ADXR condition were different from those under the sham condition (**A**: $p = 0.001$; **B**: $p = 0.04$; **C,D**: $p < 0.001$). *n*=16-21/prenatal treatment/surgical condition/sex.

Post-CUS corticosterone. In females, post-CUS basal corticosterone levels were lower overall in the ADXR than the sham condition (main effect of surgical condition: $F_{1,106} = 23.108$, $p < 0.001$; Figure 4.5**A**). Furthermore, as expected, CUS increased basal corticosterone levels in all prenatal groups under the sham condition, while there were no differences between non-CUS and CUS females under the ADXR condition (surgical condition x CUS interaction: $F_{1,106} = 3.990$, $p = 0.048$; Figure 4.5**A**).

There were no effects of prenatal treatment, surgical condition, or CUS exposure in males (Figure 4.5**C**).

Post-CUS ACTH. In females, post-CUS basal ACTH levels were higher in the ADXR than the sham condition (main effect of surgical condition: $F_{1,92} = 15.692$, $p < 0.001$; Figure 4.5**B**).

In males, both PAE and PF animals had higher post-CUS basal ACTH levels than C males (main effect of prenatal treatment: $F_{2,100} = 4.452$, $p = 0.014$). In addition, a surgical condition x CUS exposure interaction ($F_{1,100} = 8.019$, $p = 0.006$; Figure 4.5**D**) indicated that while there were no effects of CUS under the sham condition, CUS increased basal ACTH levels overall under the ADXR condition compared to both their non-CUS and sham counterparts. However, inspection of Figure 4.5**D** indicates that this effect is driven primarily by PAE and PF males.

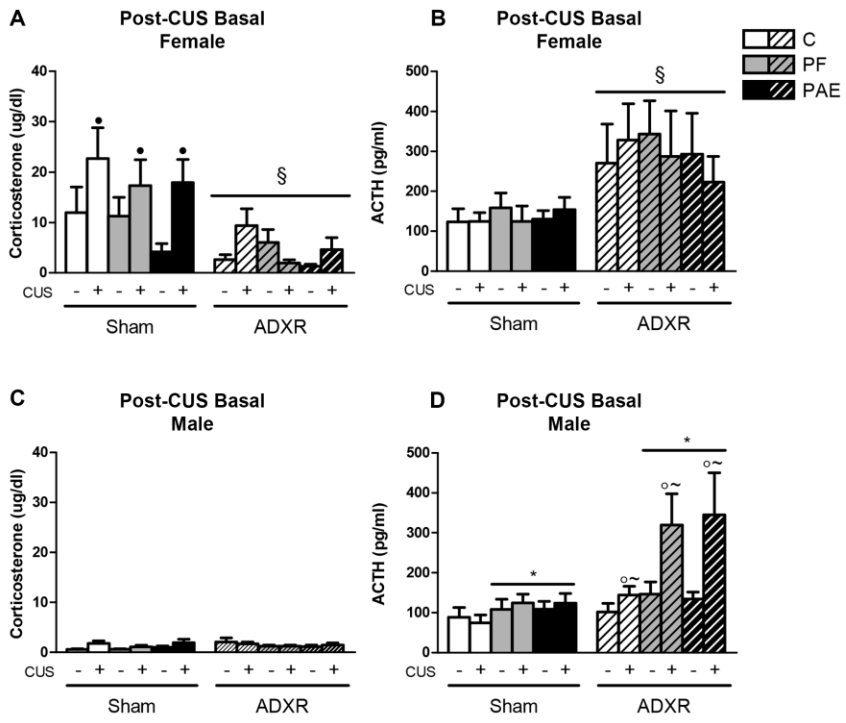

Figure 4.5 Effects of prenatal alcohol exposure (PAE), chronic unpredictable stress (CUS), and adrenalectomy with corticosterone replacement (ADXR) on post-CUS basal corticosterone (µg/dL) and ACTH (pg/ml) levels in females (A,B) and males (C,D).
Bars represent mean ± SEM. For **A**, ˙ under the sham but not ADXR condition, corticosterone levels were higher in CUS than non-CUS, regardless of prenatal groups (p = 0.001); for **A,B**, § animals under the ADXR were different from those under the sham condition (ps < 0.001); for **D**, * ACTH levels were higher overall in PF and PAE than C males (ps ≤ 0.015), and under the ADXR condition, ACTH levels were higher in CUS than their ° non-CUS and ˜ sham counterparts (ps < 0.001). In the x-axis, ⁻ indicates non-CUS while ⁺ indicates CUS animals. For A,C, *n*=9-11/prenatal treatment/surgical condition/CUS condition/sex; for B,D, *n*=6-11/prenatal treatment/surgical condition/CUS condition/sex.

4.3.4 Behavior in the open field and elevated plus maze

Open field. In females, the effects of prenatal treatment and CUS exposure on time in center depended on surgical condition (prenatal treatment x surgical condition x CUS exposure interaction: $F_{2,108} = 3.412$, p = 0.037; Figure 4.6**A**). In the sham non-CUS condition, PAE females spent more time in the center than PF females, whereas in the ADXR condition, CUS increased time in center in C females compared to their sham counterparts and decreased time in center in PF females compared to their C counterparts; however, CUS had no effects on PAE

females. Furthermore, frequency of center entries differed among prenatal treatment groups depending on surgical condition (prenatal treatment x surgical condition interaction: $F_{2,108}$ = 3.476, p =0.034; Figure 4.6**B**). Under the sham condition, PAE females entered the center more frequently than both C and PF females, with the greatest frequency of center entries in PAE females in the non-CUS conditions (*a priori* analysis, $F_{1,108}$ = 6.696, p = 0.011), whereas following ADXR, C but not PAE or PF females showed a higher frequency of center entries than their sham counterparts.

Distance travelled in the center was higher overall in PAE than PF females (main effect of prenatal treatment: $F_{2,108}$ = 4.321, p = 0.016, Figure 4.6**C**).

In males, CUS differentially affected the three prenatal treatment groups (prenatal treatment x CUS exposure interaction – time: $F_{2,110}$ = 5.835, p = 0.004, Figure 4.6**D**; entries: $F_{2,110}$ = 4.192, p = 0.018, Figure 4.6**E**; distance: $F_{2,110}$ = 4.077, p = 0.020, Figure 4.6**F**). Specifically, CUS decreased time in center, frequency of center entries, and distance travelled in the center in C males compared to their non-CUS counterparts, and increased time in center in PF animals compared to their non-CUS counterparts, while behavior of PAE males was not altered by CUS. In addition, *a priori* analyses revealed that in the sham non-CUS condition, PAE males spent less time in the center and had fewer center entries than C males (time: $F_{1,110}$ = 2.474, p = 0.047, **FIGURE 4.6D**; entries: $F_{1,110}$ = 5.716, p = 0.019, **FIGURE 4.6E**).

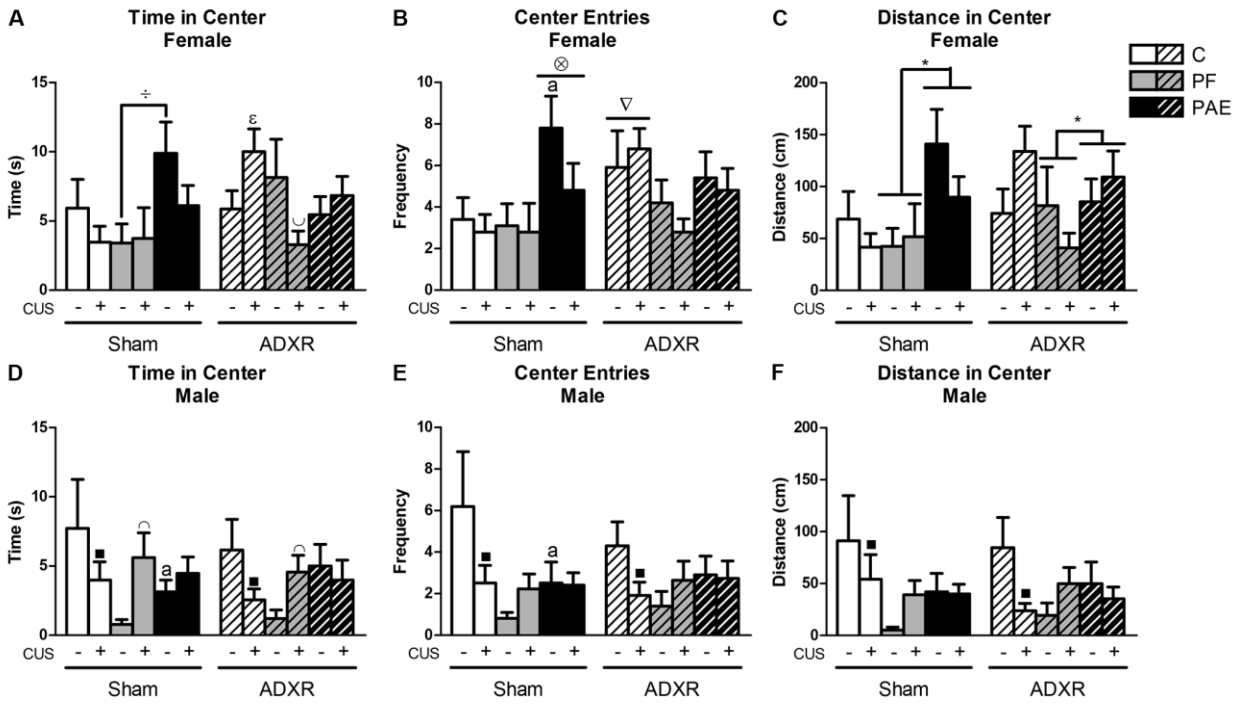

Figure 4.6 Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) and on behaviors of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats in the open field.
Bars represent the mean ± SEM of time in the center (**A,D**), frequency of center entries (**B,E**), and distance travelled in the center (**C,F**). For **A**, ÷ within the sham condition, non-CUS PAE females spent more time in the center than non-CUS PF females ($p = 0.029$); following CUS exposure, time in center was ε higher in C females in the ADXR than sham condition ($p = 0.009$), and ∪ lower in PF than C females within the ADXR condition ($p = 0.022$); for **B,D,E**, ᵃ under the sham condition, non-CUS PAE females were different from non-CUS C females (**B**: $p = 0.011$; **D**: $p = 0.047$; **E**: $= 0.019$); for **B**, ⊗ under the sham condition, PAE females entered the center more frequently than both C and PF females ($ps \leq 0.027$), while ∇ under the ADXR condition, C females showed a higher frequency of center entries than their sham counterparts ($p = 0.008$); for **C**, * distance travelled in the center was higher overall in PAE than PF females ($p = 0.004$); for **D-F**, ▪ CUS decreased time in center in C males compared to their non-CUS counterparts (**D**: $p = 0.024$; **E**: $p = 0.006$; **F**: $p = 0.016$); for **D**, ∩ CUS increased time in the center in PF males compared to their non-CUS counterparts ($p = 0.013$). In the x-axis, ⁻ indicates non-CUS while ⁺ indicates CUS animals. *n*=9-11/prenatal treatment/surgical condition/CUS condition/sex.

There were no effects of prenatal treatment, surgical condition, or CUS on total distance travelled in the field in either females and males (data not shown).

Elevated plus maze. In females, prenatal treatment and CUS exposure interacted to alter percent time on open arms (prenatal treatment x CUS exposure interaction: $F_{2,107} = 3.912$, $p = 0.023$;

Figure 4.7**A**). Specifically, under the non-CUS condition, PAE females spent more time on open arms than their PF counterparts. However, PAE but not PF or C females showed reduced time on open arms following CUS compared to their non-CUS counterparts. Overall, PAE females also entered the closed arms more frequently than both C and PF females (main effect of prenatal treatment: $F_{2,108} = 5.182$, $p = 0.007$; Figure 4.7**B**).

In males, percent time on open arms was lower overall in the ADXR than the sham condition (main effect of surgical condition: $F_{1,108} = 4.423$, $p = 0.038$; Figure 4.7**C**). As well, prenatal treatment and CUS exposure interacted to alter frequency of closed arm entries (prenatal treatment x CUS exposure interaction: $F_{2,111} = 4.038$, $p = 0.020$; Figure 4.7**D**). Under non-CUS conditions, PAE had more closed arm entries than PF males.

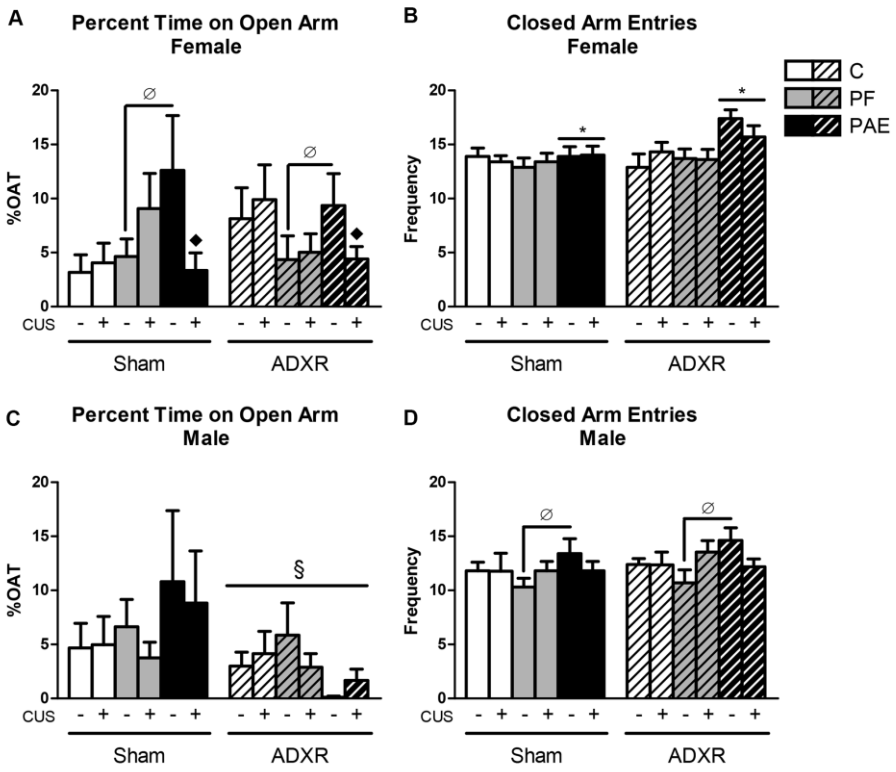

Figure 4.7 Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) and on behaviors of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats in the elevated plus maze.
Bars represent the mean ± SEM of percent time on open arms (**A,C**) and frequency of closed arm entries (**B,D**). For **A**, ∅ under the non-CUS condition, PAE females spent more percent time on open arms than their PF counterparts ($p = 0.048$), ♦ and in PAE females, percent time on open arms were lower under the CUS than non-CUS condition ($p = 0.009$); for **B**, * PAE females entered the closed arms more frequently than both C and PF females ($ps \leq 0.011$); for **C**, § males under the ADXR condition spent less percent time on open arms than those under the sham condition ($p = 0.035$); for **D**, ∅ under the non-CUS condition, PAE had more closed arm entries than PF males ($p = 0.004$). In the x-axis, $^{-}$ indicates non-CUS while $^{+}$ indicates CUS animals. *n*=9-11/prenatal treatment/surgical condition/CUS condition/sex.

4.3.5 GR and MR mRNA expression

mPFC – GR mRNA expression. In females, ADXR and CUS differentially affected PrL and IL GR mRNA expression among prenatal treatment groups (prenatal treatment x surgical condition x CUS exposure interaction – PrL: $F_{2,69} = 3.196$, $p = 0.047$, Figure 4.8**A**; IL: $F_{2,69} = 5.202$, $p = 0.008$, Figure 4.8**B**). Non-CUS C females in the ADXR condition had higher GR mRNA expression in both the PrL and IL than their sham counterparts. Moreover, under ADXR

conditions, CUS decreased GR mRNA expression in the PrL of C females compared to their non-CUS counterparts, and in the IL of C females compared to both their non-CUS and sham counterparts. This resulted in GR mRNA expression in the PrL and IL following CUS to be higher in ADXR PAE than ADXR C females (*a priori* analyses for PrL: $F_{1,69} = 5.391$, $p = 0.023$, Figure 4.8**A**; *post-hoc* comparisons for IL: $p = 0.024$, Figure 4.8**B**).

There were no effects of prenatal treatment, surgical condition, or CUS exposure in males (Figure 4.8**C,D**).

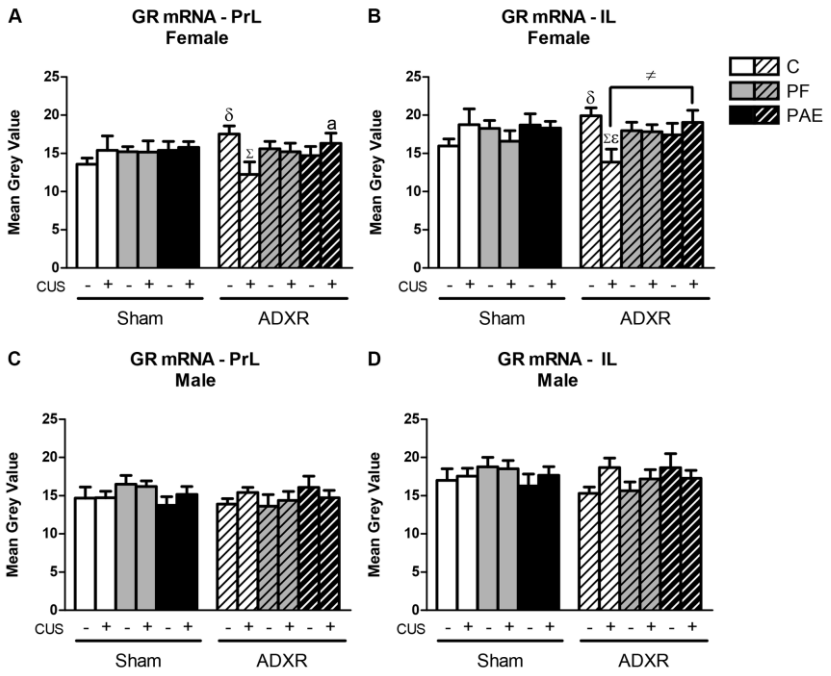

Figure 4.8 Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) on GR mRNA expression in the medial prefrontal cortex (mPFC) of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.
Bars represent mean grey values (mean ± SEM) of GR mRNA expression in the PrL (**A,C**) and IL (**B,D**) cortices of the mPFC. For **A**,**B**, $^{\delta}$ non-CUS C females in the ADXR condition had higher GR mRNA expression than their sham counterparts (**A**: p = 0.027; **B**: p = 0.041), and $^{\Sigma}$ C females in the ADXR condition had lower GR mRNA expression following CUS than their non-CUS counterparts (**A**: p = 0.005; **B**: p = 0.003); for **A**, ^{a} in the ADXR condition, GR mRNA expression following CUS was higher in PAE than C females (p = 0.023); for **B**, $^{\varepsilon}$ in C females, GR mRNA expression following CUS was lower in the ADXR than sham condition (p = 0.015), and $^{\neq}$ in the ADXR condition, GR mRNA expression following CUS was higher in PAE than C females (p = 0.024). In the x-axis, $^{-}$ indicates non-CUS while $^{+}$ indicates CUS animals. *n*=6=7/prenatal treatment/surgical condition/CUS condition/sex.

Amygdala – GR mRNA expression. There were no effects of prenatal treatment, surgical condition, or CUS exposure in either males or females in any nuclei measured (data not shown).

Hippocampal formation – GR mRNA expression. In females, ADXR increased GR mRNA expression overall in the DG, CA3, and CA1 hippocampal subregions compared to the sham condition (main effect of surgical condition – DG: $F_{1,69} = 8.448$, p = 0.005, Figure 4.9**A**; CA3: $F_{1,69} = 8.370$, p = 0.005, Figure 4.9**B**; CA1: $F_{1,69} = 8.559$, p = 0.005, Figure 4.9**C**;). In addition,

PF showed lower GR mRNA expression than C females in the CA3 and ventral subiculum, independent of CUS exposure or ADXR condition (main effect of prenatal treatment – CA3: $F_{2,69} = 3.656$, $p = 0.031$, Figure 4.9**B;** ventral subiculum: $F_{2,69} = 3.242$, $p = 0.045$, Figure 4.9**D**). In the ventral subiculum, *a priori* analyses further revealed that PAE females in the sham non-CUS condition had lower GR mRNA expression than their C counterparts ($F_{1,69} = 4.016$, $p = 0.049$; **FIGURE 4.9D**).

In males, CUS overall decreased GR mRNA expression in the CA3, independent of prenatal treatment or surgical condition (main effect of CUS exposure: $F_{1,72} = 4.405$, $p = 0.039$; Figure 4.9**F**).

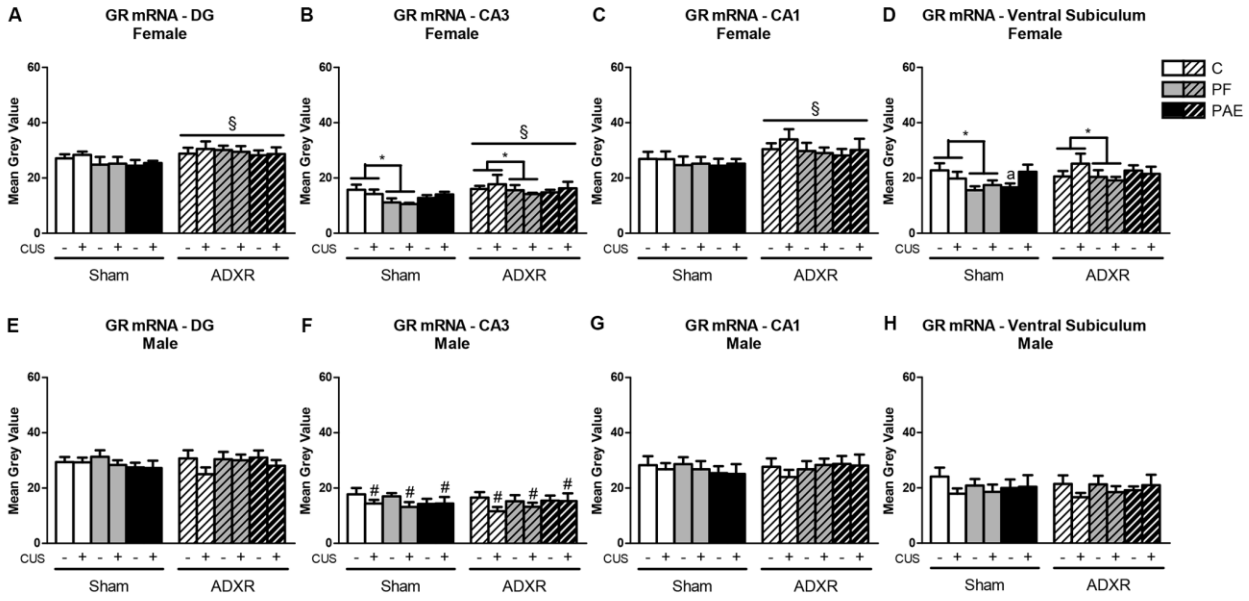

Figure 4.9 Effects of adrenalectomy with corticosterone replacement (ADXR) and chronic unpredictable stress (CUS) on GR mRNA expression in the hippocampal formation of adult male and female control (C), pair-fed (PF), and prenatal alcohol-exposed (PAE) rats.
Bars represent mean grey values of GR mRNA expression in the dentate gyrus (DG; **A,E**), CA3 (**B,F**), CA1 (**C,G**) and ventral subiculum (**D,H**) subregions of the hippocampal formation. For **A-C**, § ADXR increased GR mRNA expression compared to the sham condition (ps = 0.005); for **B,D**, * PF females showed lower GR mRNA expression than C females (**A**: p = 0.009; **B**: p = 0.015)); for **D**, [a] in the sham condition, non-CUS PAE females than their non-CUS C counterparts (p = 0.049); for **F**, [#] males under the CUS condition showed lower GR mRNA expression than their non-CUS counterpart, regardless of prenatal treatment or surgical condition (p = 0.039). In the x-axis, [−] indicates non-CUS while [+] indicates CUS animals. *n*=6-7 /prenatal treatment/surgical condition/CUS condition/sex.

Hippocampal formation – MR mRNA expression. In females, there were no effects of prenatal treatment, surgical condition, or CUS exposure on MR mRNA expression. By contrast, in males, ADXR decreased MR mRNA expression in the DG (mean grey value ± SEM of 43.7 ± 1.2 for ADXR vs 48.3 ± 1.5 for sham; main effect of surgical condition: $F_{1,72} = 5.290$, p = 0.024).

4.4 Discussion

The present study aimed to determine whether PAE-induced HPA hyperresponsivity to stress – in particular, corticosterone hypersecretion – mediates the differential effects of stress on

behavior and expression of MR and GR mRNA in PAE and C animals. We utilized ADXR to normalize corticosterone levels in adult PAE males and females, exposed them to CUS, and then challenged them in behavioral tasks that measure anxiety-like behavior and examined peripheral and central HPA activity/regulation. Replacing corticosterone by adding it to the drinking water resulted in a strong circadian rhythm and relatively low variability in corticosterone levels achieved. Consistent with our previous findings (Glavas et al., 2007, 2001), while ADXR resulted in corticosterone levels within the basal physiological range in both females and males, plasma corticosterone levels were at the lower end of the physiological range for females and were insufficient to normalize ACTH levels for both females and males. Although the ADXR procedure only approximated the sham condition, it is important to note that corticosterone intake and basal corticosterone levels were not different among prenatal treatment groups.

We found that under the non-CUS condition, PAE differentially altered anxiety-like behavior in females and males, with females showing decreased anxiety-like behavior but males exhibiting increased anxiety-like behavior compared to their C counterparts. PAE also decreased GR mRNA expression in the hippocampal formation in females but had no effects on MR or GR mRNA expression in any brain region in males. CUS had differential effects on anxiety-like behavior in PAE and C animals, and these effects were sex dependent. Moreover, ADXR unmasked differences between PAE and C animals, demonstrating that 1) corticosterone may play a differential role in modulating behavior and HPA activity/regulation in PAE and C females (Figure 4.10); 2) corticosterone may not be involved in mediating the effects of PAE and CUS on anxiety-like behavior and receptor expression in males; and 3) PAE males may be unable to appropriately utilize an exogenous corticosterone signal.

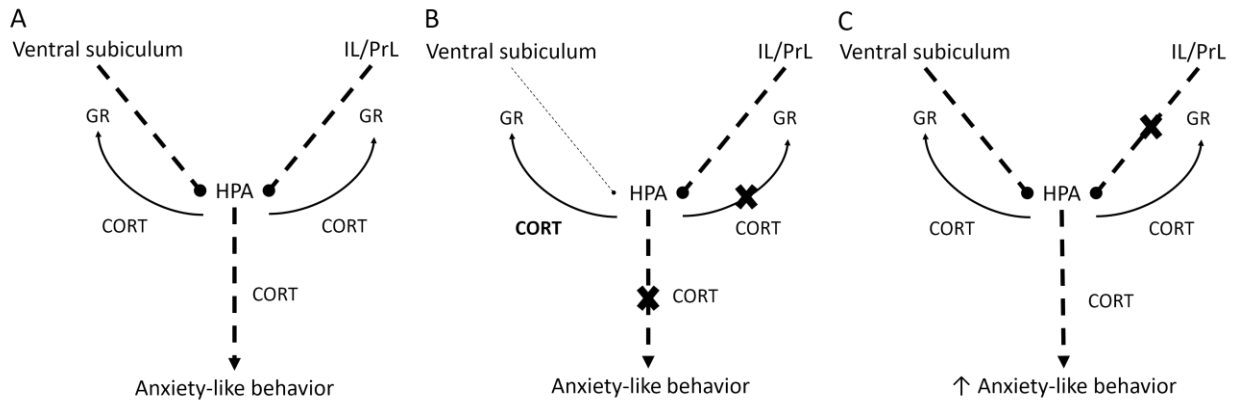

Figure 4.10 Working models to illustrate GR-mediated regulation of hypothalamic-pituitary-adrenal (HPA) function by the ventral subiculum and infra- and prelimbic cortices (IL/PrL) of the medial prefrontal cortex and downstream effects on anxiety-like behavior in control (A), prenatal alcohol-exposed (PAE; B), and prenatal alcohol- and chronic unpredictable stress (CUS)-exposed (C) females.
(A) In C females, the HPA axis secretes corticosterone (CORT), which may modulate anxiety-like behavior. The ventral subiculum and IL/PrL, indirectly, exert inhibitory effects on the HPA axis and these regions are responsive to corticosterone feedback through the presence of glucocorticoid receptors (GR). **(B)** PAE alone decreased anxiety-like behavior and decreased glucocorticoid receptor (GR) mRNA expression in the ventral subiculum. PAE also altered CORT-mediated modulation of anxiety-like behavior. In addition, GR mRNA expression in the ventral subiculum in PAE females may be mediated by elevated corticosterone levels in response to the stress of behavioral testing. Moreover, in the IL/PrL, PAE showed deficits in corticosterone-mediated GR regulation. (**C**) PAE females showed increased anxiety-like behavior following CUS exposure. Altered regulation of GR mRNA expression in the IL/PrL of PAE compared to C females was unmasked following CUS exposure under ADXR conditions. Dashed lines indicate indirect effects. Solid circles at the end of the dashed lines indicate inhibitory effects. Arrows at the end of the dashed lines indicate general modulatory effects.

4.4.1 Corticosterone may have differential roles in regulating anxiety-like behavior and GR mRNA expression in PAE and C females

In females under the non-CUS condition, sham PAE animals spent more time in the center, entered the center more frequently, and travelled greater distance in the open field, as well as spent higher percent time on open arms in the elevated plus maze than C and/or PF animals. There were no differences in locomotor activity in the open field among prenatal treatment groups, but PAE females were hyperactive in the elevated plus maze (increased frequency of

closed arm entries), regardless of surgical condition or CUS exposure. While behavior of females overall would typically be interpreted as representing decreased anxiety-like behavior, it may also be related to hyperactivity, possibly due to deficits in response inhibition, as suggested by data from other preclinical studies (Barron and Riley, 1990; Caul et al., 1979; Molina et al., 1984; Riley et al., 1979). Regardless, our finding is consistent with the literature showing that PAE either had no effects or decreased anxiety-like behavior in females in the elevated plus maze and open field (Fish et al., 2018; Gabriel et al., 2006; Hellemans et al., 2008; Osborn et al., 1998b; Staples et al., 2013).

Post-CUS basal corticosterone levels were increased in all females under the sham condition, providing support that the current CUS paradigm was of adequate intensity to effectively increase HPA activity; a hyperactive HPA axis has been suggested to be necessary and sufficient for inducing behavioral effects of CUS (Willner, 2017). Despite similar effects on basal corticosterone levels among prenatal treatment groups, the effects of CUS on behavior differed between PAE and control females. While there were no effects of CUS on behavior of C and PF females in either the open field or elevated plus maze, CUS decreased frequency of center entries in the open field and percent time on open arms in the elevated plus maze in PAE females to levels comparable to those of their control counterparts. These results can be interpreted as either CUS normalizing the behavior of PAE females to that of their C counterparts, or CUS increasing anxiety-like behavior in PAE females compared to their non-CUS behavior. The latter is in line with the diagnostic criteria outlined in the DSM-V for major depressive disorder, in which anxious distress is a prominent feature, stating that “symptoms…represent a change from previous functioning” (“Depressive disorders,” 2013). Furthermore, as the latter interpretation is

focused on the within-group effect of CUS, it is akin to how drugs are assessed for their anxiolytic or anxiogenic properties during screening – that is, whether a drug has anxiolytic or anxiogenic effects depend on the direction of changes in behavior (e.g. increased or decreased open arm time in the elevated plus maze, respectively) within rat strains and independent of differences in baseline behavior among strains (Hogg, 1996; Ramos et al., 1997). Regardless of interpretation, these results build on and extend previous findings – which demonstrate that the effects of acute stress on anxiety-like behavior may be more pronounced in PAE compared to C females (Gabriel et al., 2006; Osborn et al., 1998a) – to show that PAE females may be differentially sensitive to the effects of CUS on anxiety-like behavior compared to C females.

Our ADXR results suggest that corticosterone may have differential roles on anxiety-like behavior in PAE and C females. C females in the ADXR condition in general showed decreased anxiety-like behavior compared to their sham counterparts. However, ADXR did not have the same effect on PAE females; instead, their behavior was normalized to the levels of C females, regardless of CUS exposure. These results suggest that corticosterone levels may be involved in modulating behavior in females – with lower corticosterone levels decreasing anxiety-like behavior in C but not PAE animals – and that the role of corticosterone in behavior may potentially differ between PAE and C animals.

Under the sham condition, decreased GR mRNA expression in the ventral subiculum in non-CUS PAE compared to non-CUS C females may potentially underlie the decrease in anxiety-like behavior following PAE. The ventral subiculum has been suggested to subserve associative learning of adaptive coping strategies following a stressful or arousing context, and does so

through its involvement in context representation and connection with brain areas involved with action selection, such as the nucleus accumbens (NAc) (Floresco, 2015; Lipski et al., 2017). In particular, the ventral subiculum directly projects to the NAc, and this pathway is activated by acute stressors (Lipski et al., 2017). The ventral subiculum is also involved with feedback inhibition of the HPA axis (Herman et al., 2016). As such, alteration in the ventral subiculum-NAc connection may ultimately impact action selection in a stressful situation. GR has been suggested to modulate BDNF expression, and BDNF is involved with neuronal growth and survival, as well as in synaptogenesis and plasticity (Chen et al., 2017). Therefore, changes in GR mRNA expression under the non-CUS condition in PAE females may, at least indirectly, impact both feedback inhibition of the HPA axis and the ventral subiculum-NAc circuit, resulting in altered action selection when exposed to an acute stressor, such as the open field and elevated plus maze. It is possible that this may, at least partly, underlie the "inappropriate" decrease in anxiety-like behavior observed in PAE females.

In addition to its possible role in the behavioral alterations observed, corticosterone levels may play a role in modulating GR mRNA expression in the ventral subiculum and the mPFC in females, and corticosterone may play a differential role in PAE and C females. In the ventral subiculum, clamping corticosterone levels through ADXR normalized the aforementioned differences between non-CUS PAE and C females, by increasing GR mRNA expression in non-CUS PAE females. This finding suggests that hypersecretion of corticosterone during the stress response may be involved with regulating GR mRNA expression in PAE females. Additionally, in the PrL and IL, which are both involved with feedback inhibition of the HPA axis in response to stress (Herman et al., 2016), ADXR caused an increase in GR mRNA expression in non-CUS

C, but not PAE, females compared to their sham counterparts. These results suggest that PAE females may have deficits in corticosterone-mediated GR regulation, and consequently, negative feedback regulation of the HPA axis, compared to C females, as they failed to show the typical alterations in GR mRNA expression exhibited by their C counterparts in response to ADXR.

In addition, CUS decreased GR mRNA expression in the IL and PrL in C but not PAE females. It is puzzling, however, that this CUS effect in C females was observed under the ADXR condition where corticosterone levels were clamped in the basal physiological range. CUS has been shown to decrease GR mRNA expression in the rat frontal cortex, but GR downregulation has been postulated to be due to the hypersecretion of corticosterone following CUS (Hill et al., 2012). Our pre- and post-CUS basal corticosterone data provide support that corticosterone levels were normalized among prenatal treatment and CUS groups under the ADXR condition, albeit on the lower end of the physiological range. It is not well understood what factors may regulate transcriptional and protein expression of GR, but corticosterone-independent mechanisms that influence GR levels and GR-corticosterone interaction may exist (reviewed in (Hapgood et al., 2016)). Of particular relevance, basal immune activation status may impact GR levels as well as sensitivity and responsiveness of GR to corticosterone (Hapgood et al., 2016). PAE has been found to alter developmental cytokine levels (Bodnar et al., 2016; Drew et al., 2015); as such, corticosterone-independent mechanisms, such as those involving cytokines, may potentially contribute in part to the differences in GR mRNA expression in the mPFC in PAE and C females that were unmasked by the ADXR procedure. Nevertheless, together with findings in GR mRNA expression in the ventral subiculum, corticosterone levels may play a role

in modulating GR mRNA expression in females, but the role of corticosterone may be different between PAE and C females.

4.4.2 Corticosterone does not appear to be involved in mediating the effects of PAE and CUS on behavior and hippocampal GR mRNA expression in males

Under non-CUS conditions, sham PAE males spent less time in the center and made fewer center entries in the open field compared to sham C males, providing support for previous findings that PAE may increase anxiety-like behavior (Brocardo et al., 2012; Cullen et al., 2013; Hofmann et al., 2005; Raineki et al., 2016; Rouzer et al., 2017). However, PAE had no effects on GR and MR expression in any brain regions examined, suggesting that while PAE males may be constitutively vulnerable to the effects of the acute stress of open field testing, central regulation of HPA activity by GR and MR may not be involved in mediating this vulnerability. Interestingly, although CUS exposure had no effects on post-CUS basal corticosterone levels, it increased anxiety-like behavior (decreased time in center, frequency of center entries, and distance travelled in the center of the open field) in C males, regardless of surgical condition. In comparison, PAE males under the CUS condition exhibited similar levels of anxiety-like behavior as both their non-CUS counterparts and CUS C males, providing further support that PAE males may be constitutively susceptible to the effects of the acute stress of testing in the open field. These effects of PAE and CUS were in the context of similar locomotor activity among groups, as measured by total distance travelled in the field. The behavioral findings in C males provide support that the current CUS paradigm used was effective in inducing changes in behavior even though it had no effects on basal corticosterone levels. This is consistent with the literature, showing that while CUS typically induces an increase in basal corticosterone levels,

behavioral effects of CUS may be present without changes in corticosterone levels (reviewed in (Willner, 2017)). Consistent with findings from previous studies (reviewed in (Hill et al., 2012)), CUS also decreased GR mRNA expression in the CA3 in males, regardless of prenatal treatment. Given the role of the hippocampal formation in negative feedback of the HPA stress response (Herman et al., 2016), a CUS-induced decrease in hippocampal GR mRNA expression has implications for the HPA stress response, and may underlie the increase in anxiety-like behavior in C males following CUS.

By contrast to females, ADXR did not normalize effects of PAE or CUS exposure on behavior and GR mRNA expression, suggesting that corticosterone does not play a key role in mediating the effects of PAE or CUS in males. ADXR, however, did result in decreased percent time on open arms in the elevated plus maze and decreased MR mRNA expression in the DG compared to that in the sham condition, regardless of prenatal treatment and CUS exposure. While basal corticosterone levels post-CUS were not different among groups, levels pre-CUS were found to be higher in the ADXR than sham condition. Whether the transient increase in pre-CUS basal corticosterone levels can contribute to the long-term decrease in MR mRNA expression needs further investigation. Regardless, MR is involved with regulating basal HPA tone, setting the threshold of HPA reactivity to stress, and maintaining high neuronal excitability (Joëls and de Kloet, 2017; ter Heegde et al., 2015), as well as with promoting neurogenesis, maintaining neuronal integrity, and preventing GR-mediated apoptosis (ter Heegde et al., 2015). Moreover, high MR expression has been suggested to confer resilience to stress (ter Heegde et al., 2015). Therefore, decreased MR mRNA expression in the DG may underlie increased anxiety-like behavior in the elevated plus maze following ADXR.

4.4.3 PAE males appeared unable to appropriately utilize an exogenous corticosterone signal

While there were no effects of prenatal treatment in females, we found that post-CUS basal ACTH levels were increased in both PAE and PF compared to C males, regardless of surgical condition or CUS exposure. Furthermore, the overall increase in post-CUS basal ACTH levels observed in males was driven primarily by CUS-induced increases in ACTH in PAE and PF males under the ADXR condition. That the ADXR condition unmasked higher ACTH levels in PAE compared to C males following CUS indicates that corticosterone replacement at the same levels in all prenatal treatment groups was sufficient to normalize HPA activity in C but not PAE males, providing support for the previous suggestion that PAE males may have decreased sensitivity to negative feedback regulation by corticosterone (Glavas et al., 2007). Furthermore, previous findings have demonstrated that PAE males show increased HPA drive (Glavas et al., 2007, 2001; Lan et al., 2015) and increased pituitary sensitivity to CRH following dexamethasone suppression (Osborn et al., 2000) compared to C males, which together with decreased sensitivity to corticosterone-mediated negative feedback, likely contribute as well to the observed higher basal ACTH levels following CUS in PAE than C males. We also found that PAE males in the ADXR condition showed attenuated weight gain post-CUS compared to their C and sham counterparts, regardless of CUS condition. This may indicate that PAE males may need a longer time than their control counterparts to recover from an invasive surgery such as ADX. Alternatively, it may indicate that PAE males may not be able to adequately utilize the exogenous corticosterone provided compared to their control counterpart. One of the functions of corticosterone is on metabolism of nutrients and corticosterone can influence body weight gain via activations of both peripheral and central MRs and GRs (Devenport et al., 1989; Tempel and

Leibowitz, 1994). Our findings of elevated post-CUS basal ACTH levels and reduced body weight gain under the ADXR condition suggest the possibility that PAE males may be unable to appropriately utilize an exogenous corticosterone signal.

4.4.4 PAE effects are different from pair-feeding effects

While certain results were similar between PAE and PF animals, including post-CUS body weight changes and basal ACTH levels in males, we found that PAE differed from PF animals in their behavior in the open field and elevated plus maze. A PF group is included to serve as a control for the reduction in food intake typically observed in alcohol-consuming animals. PF animals receive a reduced ration of a nutritionally optimal diet matched in amount to a PAE partner (Weinberg, 1985, 1984). However, pair-feeding cannot control for other nutritional effects of alcohol, such as effects on nutrient utilization and absorption. There is also a component of mild prenatal stress involved with receiving a reduced food ration, as these animals are given less food than they would otherwise consume if they had *ad libitum* access; therefore, the entire ration is typically consumed within a few hours of diet presentation and the PF animals are food-deprived and hungry until feeding the next day. Therefore, the PF animals form an imperfect control group. Regardless, our findings indicate that while GR and MR mRNA expression may be similar between PAE and PF animals, PAE induces different effects on anxiety-like behavior. As such, effects of PAE are not simply due to a reduction in food consumption, but rather, unique to the effects of alcohol exposure during the prenatal period.

4.4.5 Limitations

While we were able to localize differential MR and GR mRNA expression in specific nuclei and reveal potential neurobiological underpinnings for behavioral changes following PAE and CUS, we did not measure changes in protein levels and function. Although findings from a recent study provide support for the implicit assumption that protein levels would correspondingly change with alterations in mRNA expression (Koussounadis et al., 2015), the translation of mRNA to protein involves intricate coordination among multiple molecular players, and PAE effects on these components have not been examined. Furthermore, mRNA expression results cannot account for post-translational modifications of proteins that can impact functional outcome by altering activity, stability, amount, localization, and protein-ligand and protein-protein interactions. PAE may exert influences on processes downstream of transcription, which can in turn impact behavioral output; therefore, further experimentation is warranted. Nevertheless, our findings can serve as a guide for future experiments that directly examine the relationship between changes in expression of specific genes and behavioral outcome.

Another limitation in the present experimental design is that multiple behavioral tests were conducted in the same animal without counterbalancing for order of testing. Behavioral testing involves a component of stress, and should be considered as a manipulation as well. The order of testing can impact behavioral responses, and the placement of a more stressful behavioral test, such as FST, in the line-up of tests needs careful consideration (Blokland et al., 2012; McIlwain et al., 2001). Our typical practice is to order the tests from least to most stressful, e.g., FST is typically performed last. The possibility that PAE animals may respond differently from controls to the cumulative stress from a particular testing order remains to be determined and could

provide additional insight into the outcomes we observed. As it stands, the present study does provide important information on brain and behavioral responses to the same cumulative stress experience (CUS + behavioral testing), which has implications for the response to stress in general in individuals prenatally exposed to alcohol. It would be an interesting extension to the current findings to probe for possible prenatal treatment x order of testing x CUS exposure interactions to further evaluate the impact of PAE on anxiety-like behavior, which could enhance interpretations of the present results.

Additionally, that testing occurred during the light phase in the present study may affect the behavioral results, and consequently, the receptor mRNA expression findings. Hormone measurements were also assessed during the light phase. It has been found that the circadian phase differentially affects behavior of male and female rats, with males showing greater anxiety-like behavior during the light than dark phase, but females showing greater anxiety- and depressive-like behavior during the dark than light phase (Verma et al., 2010). Therefore, testing exclusively during the dark phase may mask differences between experimental treatment groups on behavioral and endocrine measures (Verma et al., 2010). It would be a helpful extension to the current studies to compare behavior, hormone, and brain measures assessed in the dark vs. the light phase, which could enrich the understanding of the present results.

4.5 Conclusion

Under the non-CUS condition, PAE differentially altered anxiety-like behavior and GR mRNA expression in females and males. CUS had differential effects on anxiety-like behavior in PAE and C animals, and these effects were sex dependent. Importantly, our ADXR findings suggest

that corticosterone levels may be involved in mediating the effects of PAE on anxiety-like behavior and GR mRNA expression in females, but not males, and that the role of corticosterone on anxiety-like behavior and HPA regulation differs, in a sex-dependent manner, between PAE and C animals. Moreover, PAE males appeared to be unable to appropriately utilize an exogenous corticosterone signal. While HPA hyperreactivity to stress is a robust consequence of PAE, we demonstrate here that the level of corticosterone, the end product of the HPA axis, may be relevant for the behavioral outcome of PAE females but not males. Overall, our findings have important implications for understanding the role of stress and hormone secretion in the adverse effects of PAE and for developing sex-specific interventions and treatments for individuals prenatally exposed to alcohol.

Chapter 5: Overall Discussion and Conclusions

5.1 Overall discussion across the studies

First acknowledged about 50 years ago that prenatal alcohol exposure has detrimental consequences on the offspring (Jones and Smith, 1973; Lemoine et al., 1968), FASD is often viewed as a developmental disorder with research to date focusing on the early life period, and studies on the outcomes of adolescents and adults with FASD remain sparse (Moore and Riley, 2015). Additionally, in the absence of good information, it can be challenging to identify at-risk individuals early and provide timely interventions (Murawski et al., 2015). Therefore, there is a need for clinical and preclinical studies on later life outcome to help individuals in adolescence and adulthood who were not diagnosed early in life or had received a mis-diagnosis. Delineating the mechanisms by which PAE and stress (CUS), independently and interactively, may contribute to the development of mental health issues and other adverse outcomes will ultimately help establish novel and sex-specific interventions and treatments for individuals prenatally exposed to alcohol.

Body weight

The work presented in this dissertation highlights the long-lasting impact of alcohol exposure during prenatal development on neurobiology and behavior in response to chronic stress later in life. **TABLE 5.1** summarizes all the findings in males while **TABLE 5.2** summarizes all the findings in females in Chapters 2 to 4. We found that although PAE did not significantly alter the birthweight and body weight of our animals from birth until weaning, we did observe slightly lower birthweight in PAE animals, and a significant increase in body weight compared to control

counterparts during adulthood. As summarized in **TABLES 5.1 and 5.2**, adult PAE males were somewhat or significantly heavier than control males both before and after surgery, while adult PAE females had higher body weight than their control counterpart prior to CUS or surgery. Through the lens of the "developmental or fetal origins" hypothesis of health and disease, low birthweight and accelerated postnatal growth have been suggested to be associated with significant risk for adult obesity, cardiovascular disease, and metabolic syndrome, and the relevance of this hypothesis has recently been extended to include effects on other adult risks such as immune function and mental health (Lau et al., 2011). This hypothesis is related to the "thrifty phenotype" and "match-mismatch" hypotheses which suggest that the prenatal environment predicts the availability of resources in the postnatal environment, with nutrient scarcity during the prenatal period resulting in the programming of a "thrifty phenotype" set to be adaptive to conserve nutrients in a predicted deprived postnatal environment (Lau et al., 2011). This type of programming would then be maladaptive in a postnatal environment with adequate or abundant nutrition (Weinberg, 2016). Our finding that PAE animals were born slightly smaller than their control counterparts, together with accelerated postnatal growth may serve as an indicator of fetal programming and the altered quality of the intrauterine environment.

MALES (with intact adrenals)

a) Body weight	PAE	CUS-1	CUS-14	Non-CUS C	Non-CUS PAE	CUS-1 C	CUS-1 PAE	CUS-14 C	CUS-14 PAE
Body weight – PND 1 (Ch 2&3)	-								
Body weight – PND 1 (Ch 4)	-								
Body weight – PND 8 (Ch 2&3)	-								
Body weight – PND 8 (Ch 4)	-								
Body weight – PND 15 (Ch 2&3)	-								
Body weight – PND 15 (Ch 4)	-								
Body weight – PND 22 (Ch 2&3)	-								
Body weight – PND 22 (Ch 4)	-								
Body weight – pre-CUS (Ch 2&3)	-								
Body weight – pre-surgery (Ch 4)	-								
Body weight – pre-CUS (Ch 4)	↑ (C)								
Change in body weight post-CUS (Ch 2&3)	-	less weight gain (non-CUS)		-	-	-	-	-	-
Change in body weight post-CUS (Ch 4)	-	less weight gain (non-CUS)		-	-	-	-		
b) Hormones									
Corticosterone – pre-CUS (Ch 2&3)	-								
Corticosterone – pre-CUS (Ch 4)	-								
ACTH – pre-CUS (Ch 4)	-								
Corticosterone – post-CUS (Ch 2&3)	-	↑ (non-CUS)		-	-	-	-	-	-
Corticosterone – post-CUS (Ch 4)	-	-		-	-	-	-		
ACTH – post-CUS (Ch 4)	↑ (C)	-		-	-	-	-		
c) Anxiety- and Depressive-like Behavior									
Open field (Ch 2&3)	-	-	-	-	-	-	-	-	-
Open field (Ch 4)	-	-		-	↓ center time, ↓ center entries (non-CUS C)	↓ center time, ↓ center entries, ↓ center distance (non-CUS C)	-		
Elevated plus maze (Ch 2&3)	-	-	-	-	-	-	-	-	-
Elevated plus maze (Ch 4)	-	-		-	↑ closed arm entries (non-CUS PF)	-	-		
Dark-light emergence (Ch 2&3)	↓ time in light (C)	-	-	-	-	-	-	-	↑ latency to emerge (CUS-14 C)

MALES (with intact adrenals)									
Forced swim test – day 1 (Ch 2&3)	-	↓ immobility, ↑ swimming (non-CUS)		-	-	-	↓ immobility, ↑ swimming (CUS-1 C)	-	-
Forced swim test – day 2 (Ch 2&3)	-	↓ immobility, ↑ swimming, ↑ climbing (non-CUS)		-	-	-	-	-	-
d) mRNA expression									
c-fos mRNA (Ch 2&3)	-	-	-	-	↓ lateral, ↓ central (non-CUS C)	-	-	-	↑ CA1 (CUS-14 C)
CRHR1 mRNA (Ch 2&3)	-	↑ lateral, ↑ medial (non-CUS, CUS-14)	-	-	-	-	↓ IL (CUS-1 C)	-	-
MR mRNA (Ch 2&3)	-	-	-	-	-	-	-	-	-
MR mRNA (Ch 4)	-	-		-	-	-	-		
GR mRNA (Ch 2&3)	↓ medial (C)	↑ IL, ↑ lateral, ↑ basal, ↑ central, ↑ medial (CUS-14)	-	-	-	-	↓ lateral, ↓ basal, ↓ central (CUS-1 C)	-	-
GR mRNA (Ch 4)	-	↓ CA3 (non-CUS)		-	-	-	-		
MALES (ADXR)									
a) Body weight	PAE	CUS-1	CUS-14	Non-CUS C	Non-CUS PAE	CUS-1 C	CUS-1 PAE	CUS-14 C	CUS-14 PAE
Change in body weight post-CUS	less weight gain (C)	less weight gain (non-CUS)		-	-	-	-		
b) Hormones									
Basal corticosterone – pre-CUS	-								
Basal corticosterone – post-CUS	-	-		-	-	-	-		
Basal ACTH – pre-CUS	-								
Basal ACTH – post-CUS	↑ (C)	-		-	-	-	↑ (sham non-CUS PAE & ADXR non-CUS PAE)		

MALES (ADXR)									
c) Anxiety- and Depressive-like Behavior									
Open field	-	-		-	-	↓ center time, ↓ center entries, ↓ center distance (ADXR non-CUS C)	-		
Elevated plus maze	-	-		-	↑ closed arm entries (ADXR non-CUS PF)	-	-		
d) mRNA expression									
GR mRNA	-	↓ CA3 (non-CUS)		-	-	-	-		
MR mRNA	-	-		-	-	-	-		

Table 5.1 Summary of findings in males on the effects of prenatal alcohol exposure (PAE) and/or chronic unpredictable stress (CUS) on growth (i.e. body weight), hormones, depressive- and anxiety-like behavior, and mRNA expression across Chapters 2 to 4.
The comparison group against which differences were found is indicated in brackets.

FEMALES (with intact adrenals)

a) Body weight	PAE	CUS-1	CUS-14	Non-CUS C	Non-CUS PAE	CUS-1 C	CUS-1 PAE	CUS-14 C	CUS-14 PAE
Body weight – PND 1 (Ch 2&3)	-								
Body weight – PND 1 (Ch 4)	-								
Body weight – PND 8 (Ch 2&3)	-								
Body weight – PND 8 (Ch 4)	-								
Body weight – PND 15 (Ch 2&3)	-								
Body weight – PND 15 (Ch 4)	-								
Body weight – PND 22 (Ch 2&3)	-								
Body weight – PND 22 (Ch 4)	-								
Body weight – pre-CUS (Ch 2&3)	↑ (C)								
Body weight – pre-surgery (Ch 4)	↑ (C)								
Body weight – pre-CUS (Ch 4)	-								
Change in body weight post-CUS (Ch 2&3)	-	weight loss (non-CUS)		-	-	-	-	-	-
Change in body weight post-CUS (Ch 4)	-	-		-	-	-	-		
b) Hormones									
Corticosterone – pre-CUS (Ch 2&3)	-								
Corticosterone – pre-CUS (Ch 4)	-								
ACTH – pre-CUS (Ch 4)	-								
Corticosterone – post-CUS (Ch 2&3)	-	-		-	-	-	-	-	-
Corticosterone – post-CUS (Ch 4)	-	↑ (non-CUS)		-	-	-	-		
ACTH – post-CUS (Ch 4)	-	-		-	-	-	-		
c) Anxiety- and Depressive-like Behavior									
Open field (Ch 2&3)	↓ center entries (C)	↓ center entries, ↓ center time, ↓ center distance (non-CUS); ↑ latency to center (non-CUS)	↓ total distance (non-CUS)	-	-	-	-	-	-
Open field (Ch 4)	↑ center distance (PF)	-		-	↑ center time (non-CUS PF); ↑ center entries, (non-CUS C)	-	-		
Elevated plus maze (Ch 2&3)	-	-	-	-	-	-	-	-	-
Elevated plus maze (Ch 4)	↑ closed arm entries (C)	-		-	↑ % OA time (PF)	-	↓ % OA time (non-CUS PAE)		
Dark-light emergence (Ch 2&3)	-	-	-	-	-	-	-	-	-

FEMALES (with intact adrenals)									
Forced swim test – day 1 (Ch 2&3)	-	-	-	-	-	-	-	-	-
Forced swim test – day 2 (Ch 2&3)	-	↑ immobility, ↓ swimming (non-CUS)	-	-	-	-	-	-	↑ immobility, ↓ climbing (CUS-14 C)
d) mRNA expression									
c-fos mRNA (Ch 2&3)	↓ Cg1 (C)	-	↓ Cg1, ↓ PrL (non-CUS & CUS-1); ↓ IL (non-CUS)	-	-	-	-	-	-
CRHR1 mRNA (Ch 2&3)	-	↑ IL (CUS-14)	-	-	-	-	-	-	-
MR mRNA (Ch 2&3)	-	-	↓ DG (non-CUS)	-	-	-	-	-	↑ CA3 (CUS-14 C)
MR mRNA (Ch 4)	-	-		-	-	-	-		
GR mRNA (Ch 2&3)	↓ IL	-	↓ lateral, ↓basal (non-CUS, CUS-1); ↓ central (non-CUS)	-	-	-	-	-	-
GR mRNA (Ch 4)	-	-		-	↓ ventral subiculum (non-CUS C)	-	-		
FEMALES (ADXR)									
a) Body weight	PAE	CUS-1	CUS-14	Non-CUS C	Non-CUS PAE	CUS-1 C	CUS-1 PAE	CUS-14 C	CUS-14 PAE
Change in body weight post-CUS	-	-		-	-	-	-		
b) Hormones									
Basal corticosterone – pre-CUS	-								
Basal corticosterone – post-CUS	-	-		-	-	-	-		
Basal ACTH – pre-CUS	-								
Basal ACTH – post-CUS	-	-		-	-	-	-		
c) Anxiety- and Depressive-like Behavior									
Open field	↑ center distance (ADXR PF)	-		-	-	↑ center time (sham CUS-1 C)	-		
Elevated plus maze	↑ closed arm entries (ADXR C)	-		-	↑ % OA time (ADXR PF)	-	↓ % OA time (ADXR non-CUS PAE)		

FEMALES (ADXR)									
d) mRNA expression									
GR mRNA	-	-		↑ IL (sham non-CUS C)	-	↓ IL (sham CUS-1 C, ADXR non-CUS C, ADXR CUS-1 PAE)	-		
MR mRNA	-	-		-	-	-	-		

Table 5.2 Summary of findings in females on the effects of prenatal alcohol exposure (PAE) and/or chronic unpredictable stress (CUS) on growth (i.e. body weight), hormones, depressive- and anxiety-like behavior, and mRNA expression across Chapters 2 to 4.
The comparison group against which differences were found is indicated in brackets.

In addition to PAE effects, we found that in adulthood, CUS differentially affected weight gain in males and females. In males, we found consistent effects of CUS, resulting in less weight gain regardless of the minor differences in CUS paradigms used. In females, however, we observed either weight loss or no effects following CUS. The finding of weight loss in females differs from findings from other laboratories that showed that females generally appeared to be less sensitive than males to the effects of chronic stress on body weight (Dalla et al., 2005; Dunčko et al., 2001; Eiland et al., 2012; Konkle et al., 2003; Westenbroek et al., 2003). However, this finding is consistent with our previous results (Hellemans et al., 2008; Uban et al., 2013). That CUS caused weight loss in females in the studies in Chapters 2 and 3 but not in Chapter 4 may be due to differences in the age of exposure and the slightly different CUS paradigms used in the two studies (modifications were made to the CUS paradigm in Chapter 4 to accommodate the needs of animals that underwent ADX). Nevertheless, that either *less* weight gain or weight *loss* was observed with a shorter 10-day CUS paradigm, similar to what has been observed with longer multi-week paradigms (Dalla et al., 2005; Dunčko et al., 2001; Eiland et al., 2012; Konkle et al., 2003; Westenbroek et al., 2003), suggests that shorter paradigms such as ours may be useful in preclinical depression and anxiety research.

Hormones

PAE had no effects on basal corticosterone levels pre-CUS in either males (**TABLE 5.1**) and females (**TABLE 5.2**) across Chapters 2 to 4, which is consistent with the general finding that basal corticosterone levels are typically similar between PAE and C animals (Hellemans et al., 2010a). Additionally, PAE had no differential effects on basal corticosterone levels post-CUS, which replicates our previous findings (Hellemans et al., 2008; Uban et al., 2013). The two

different CUS paradigms, however, had differential effects on post-CUS basal corticosterone levels. The CUS paradigm used in Chapters 2 and 3 induced an increase in basal corticosterone levels post-CUS in males but not females, independent of prenatal treatment group, while the CUS paradigm used in Chapter 4 induced an increase in basal corticosterone levels post-CUS in females but not males. Furthermore, analysis of basal ACTH levels revealed that PAE males, but not females, had higher levels than controls, which is consistent with previous studies that demonstrate increased HPA drive in PAE males (Glavas et al., 2007, 2001; Lan et al., 2015) and increased pituitary sensitivity to CRH following dexamethasone suppression (Osborn et al., 2000) compared to C males. Together, these findings suggest that males and females may be differentially sensitive to minor differences in the two CUS paradigms utilized in our studies, and this differential sensitivity may contribute to the variations of CUS effects on behavioral and brain measures across these studies (discussed below).

Anxiety- and depressive-like behavior

With regards to anxiety-like behavior in the open field, elevated plus maze, and dark-light emergence tests, PAE alone appears to alter anxiety-like behavior in both males and females, albeit in different behavioral tests, and CUS may exacerbate the effects of PAE and cause greater anxiety-like behavior in these animals (summarized in **TABLES 5.1 and 5.2**). However, differences in testing parameters may have influenced in results in each test across the studies. In the open field, under the more aversive testing condition of studies in Chapter 4 compared to Chapter 3 – bright vs dim lighting – PAE males showed increased anxiety-like behavior under non-CUS conditions that was comparable to how C males behaved following CUS. In the elevated plus maze, under the presumably less aversive testing condition of studies in Chapter 4

compared to Chapter 3 – each arm might be perceptively wider and less threatening for the younger and smaller animals of Chapter 4 than for the older and bigger animals of Chapters 3 – non-CUS PAE males again exhibited increased anxiety-like behavior that was comparable to the behavior of C males following CUS. PAE males, independently and with delayed testing following CUS, also exhibited increased anxiety-like behavior compared to their control counterparts in the dark-light emergence test that was utilized only in Chapter 3. By contrast, in females, in the open field under the less aversive lighting condition of Chapter 3, PAE animals exhibited increased anxiety-like behavior regardless of CUS exposure. Under the more aversive testing condition of Chapter 4, however, non-CUS PAE females appeared to exhibit *decreased* anxiety-like behavior (which may alternatively be due to deficits in response inhibition, as discussed in Chapter 4) compared to their control counterparts; importantly, CUS increased anxiety-like behavior in these females but not in controls. These same PAE females in the studies of Chapter 4, but not those in Chapter 3, also showed decreased anxiety-like behavior in the elevated plus maze compared to controls, but they were again differentially affected by CUS and showed increased anxiety-like behavior compared to their non-CUS counterparts. There were no effects of PAE or CUS in the dark-light emergence test in females.

Nuances in the psychological construct that is measured by each of the three tests may also contribute to why PAE and CUS effects were not consistently observed across all three tests. Each behavioral test likely involves subtle differences in the type of decision and risk assessment that need to be made. For the open field, there is no well protected zone like the dark compartment in the dark-light emergence test; ethologically, a rat is less likely to be preyed on in the covered, dark compartment of the dark-light emergence apparatus than it is in the periphery

of the open field. Furthermore, the open field, unlike the dark-light emergence test, has no defined boundary between the "safe" periphery and the "threatening" center. Therefore, the motivation behind the decision to leave the well-protected dark compartment of the dark-light emergence test is likely different from that for leaving the periphery of the open field. As for the elevated plus maze, compared to both the open field and dark-light emergence test, the rat must now choose between staying in the arm that it is currently residing in or investigating the three other arms from a vulnerable center zone. Therefore, while all three tests examined the unconditioned response of rats in situations where there is a conflict between exploring novel environments and avoiding open spaces (Griebel and Holmes, 2013), the underlying psychological construct may be different.

In addition to increased anxiety-like behavior, increased depressive-like behavior was observed in PAE females with delayed testing post-CUS. In males, however, immediate testing following CUS decreased immobility on day 1 of the forced swim test in PAE animals, suggesting decreased depressive-like behavior. These findings are consistent with our previous findings (Hellemans et al., 2010b), and we now extend these findings to show that the effects of CUS can also be temporally dynamic depending on the sex of the animal. Unexpectedly though, whereas immediate testing following CUS increased depressive-like behavior in females, regardless of prenatal treatment group, both immediate and delayed testing following CUS *decreased* depressive-like behavior in males. In the literature, chronic stress mostly causes an increase in immobility, at least in the short-term; however, a few studies have actually reported a decrease in immobility in the forced swim test following chronic stress (reviewed in (Willner, 2005)). Although the findings that chronic stress may decrease immobility in the forced swim test is

infrequent, it is possible that there may be research groups who have used chronic stress but did not publish their findings (Willner, 2017), and that there may be under-reporting of opposite effects of chronic stress on the forced swim test. Regardless, variation in the effects of chronic stress on behavior in the forced swim test may be influenced by differences in stress procedures used (e.g. the duration of exposure and types of stressors), the parameters of testing (e.g. the temperature of the water, size of apparatus, and depth of water) (Detke and Lucki, 1996; Slattery and Cryan, 2012), as well as strain of the subjects (Bielajew et al., 2003). Sex of the animal may also play a role, as we and others have previously found that chronic stress induces differential alterations in immobility behavior and reward sensitivity to sucrose in males and females (Bielajew et al., 2003; Dalla et al., 2011; Hellemans et al., 2010a; Sachs et al., 2014). Additionally, that each animal was assessed consecutively in multiple behavioral tests in the studies in this dissertation may have an impact on the results; the order of testing has been found to impact certain behavioral responses (Blokland et al., 2012; McIlwain et al., 2001). Therefore, both the parameters and order of testing chosen for studies in the present dissertation may potentially contribute, at least in part, to our finding that regardless of prenatal treatment group, immediate testing post-CUS increased immobility in females, while CUS overall decreased immobility in males.

mRNA expression

Males

In the male brain, non-CUS PAE males showed decreased neural activation, as measured by *c-fos* mRNA expression, in the lateral and central nuclei of the amygdala compared to non-CUS C males (**TABLE 5.1**). Activation of the amygdala is generally involved with stimulating and

promoting HPA activity. Whether this PAE-induced decrease in neural activation in the amygdala is functionally/behaviorally adaptive or maladaptive is inconclusive in the present studies; nevertheless, we identify here a potential deficit in amygdala-mediated response to stressors following PAE in males that may have potential implications for emotion and stress regulation.

With delayed testing post-CUS, neural activation in the CA1 of the hippocampal formation was increased in PAE males compared to their respective control counterpart (**TABLE 5.1**). As previously discussed in Chapter 2, we may have unmasked a potential deficit in PAE compared to C males in being able to appropriately modulate hippocampal activity following CUS exposure. Given that CA1 is a major output site of the hippocampus (Cenquizca and Swanson, 2007), changes in this subregion may have important implications for its downstream targets as well as for behavior; for one, it is possible that this PAE-induced change may contribute, at least in part, to the increased anxiety-like behavior in the dark-light emergence test in PAE males with delayed testing post-CUS. Further investigations would help elucidate the functional consequence of this PAE effect.

In males, PAE consistently had no effects on MR mRNA expression between the studies in Chapters 3 and 4, but impacted CRHR1 and GR mRNA expression in the IL of the mPFC and several nuclei of the amygdala (**TABLE 5.1**). As discussed in Chapter 3, we found that PAE males failed to show the typical upregulation of CRHR1 and GR mRNA expression in these brain areas shown by their control counterparts following immediate testing post-CUS. Although these effects were not replicated in Chapter 4, possibly due to differences in the CUS paradigms

and behavioral testing conditions between the studies (behavioral testing induces stress on the animals and may constitute an experimental manipulation), it was revealed that there may also be deficits in modulating GR mRNA expression in the CA3 of the hippocampal formation in PAE males following immediate testing post-CUS. Whether PAE-induced deficits in CRHR1 and GR mRNA expression may play a role in behavior requires further examination. Regardless, our findings suggest that PAE males may have deficits in modulating CRHR1- and GR-mediated HPA feedback and feedforward regulation, and in receptor autoregulation that may be unmasked with CUS exposure.

Females

In females (**TABLE 5.2**), PAE decreased neural activation in the Cg1 of the mPFC compared to controls. PAE also induced potential deficits in modulating downregulation of MR in the CA3 of the hippocampal formation compared to their control counterpart, which was revealed with delayed testing following CUS. Additionally, in Chapter 3, we found that PAE decreased GR mRNA expression in the IL of the mPFC. While this finding was not replicated in Chapter 4, potentially due to different parameters during behavioral testing, we found instead that GR mRNA expression in the ventral subiculum was decreased in PAE females under the non-CUS condition. A commonality between these findings is that these brain areas are involved with negative feedback regulation of the HPA axis. Furthermore, that both MR and GR mRNA expression are affected by PAE suggests alteration of the HPA axis under both basal and stress conditions. While some anxiety- and depressive-like behavior were concomitantly changed in PAE females, further investigations are needed to better understand the functional implications of these PAE effects.

Overall, PAE alone altered baseline anxiety- and depressive-like behavior as well as neural activation and stress-related receptor expression in central brain regions involved with both emotional and stress regulation compared to control, unexposed animals. PAE effects on the brain were widespread, inducing potential deficits in receptor autoregulation in brain areas involved with both drive and negative feedback of HPA activity. Additionally, differential sensitivity to the effects of chronic stress were found between unexposed animals and those prenatally exposed to alcohol. Furthermore, we extend previous findings to demonstrate that several effects of CUS may have a delayed onset, providing a more comprehensive and clinically relevant understanding of the effects of chronic stress in the context of PAE. Additionally, sex differences were found in almost all measures examined, suggesting that different mechanisms may underlie anxiety-/depressive-like behavior in males and females, and underscoring the importance of examining sex differences in brain and behavioral measures. Moreover, while PF animals may in a few cases show similar changes in brain and behavioral outcomes as PAE animals, in other cases they exhibit different neural activation patterns, mRNA expression, and behavior, indicating that while some effects of PAE and PF may overlap, the underlying mechanisms likely differ rather than represent a continuum of effects along the same pathway.

I then more directly examined the role of HPA hyperresponsivity to stress – in particular, hypersecretion of corticosterone – in mediating the effects of PAE and CUS on brain and behavior, by clamping the corticosterone response to stress at basal physiological levels via adrenalectomy followed by corticosterone replacement (ADXR) in the drinking water, a procedure that can approximate the circadian rhythm of corticosterone. Our ADXR findings (**TABLE 5.2**) show that corticosterone may be involved in mediating the effects of PAE on

anxiety-like behavior and GR mRNA expression in females and that the role of corticosterone on behavior and HPA regulation differs, in a sex-dependent manner, between PAE and C animals. Moreover, PAE males may have decreased sensitivity to negative feedback regulation by an exogenous corticosterone signal (**TABLE 5.1**). Taken together, our results suggest that while HPA hyperreactivity to stress is a robust consequence of PAE, corticosterone levels may be differentially relevant for behavioral outcome and HPA regulation of PAE males and females. Overall, our findings have important implications for understanding the role of stress and hormone secretion in the adverse effects of PAE, which has clinical relevance as individuals prenatally exposed to alcohol are at a higher risk than unexposed individuals of encountering stressful environments during their lifetimes (O'Connor and Paley, 2006; Streissguth et al., 2004, 1991).

5.2 Limitations

An aspect of the present dissertation examined the effects of PAE and CUS on depressive-/anxiety-like behavior. Our findings from a battery of behavioral tests represent an ethological-based perspective on the interactive effects of PAE and CUS on anxiety-/depressive-like behavior. The use of multiple tests of anxiety-like behavior strengthened our results, because different aspects of anxiety-like behavior and associated neurobiological underpinnings may be measured by each test (Bourin, 2015; McCormick and Green, 2013; Steimer, 2011; van der Staay et al., 2009; van Gaalen and Steckler, 2000). By contrast, our examination of PAE and CUS effects on depressive-like behavior could benefit from tests of anhedonia (e.g. sucrose preference test), which is a key symptom of depression. However, sucrose and saccharin appear to dampen the HPA axis, reducing its tone and response to stress (Ulrich-Lai, 2016), and thus the

inclusion of this test would confound our results related to HPA regulation. Investigation of anhedonia following PAE and CUS in a separate cohort of animal is warranted.

In general, behavioral tests are commonly evaluated by three criteria: predictive validity (effective treatments in humans reverse symptoms in animals), face validity (comparable symptoms between animals and humans), and construct validity (causes and risk factors that induce symptoms are similar between animals and humans) (Krishnan and Nestler, 2011; McKinney and Bunney, 1969; Schmidt, 2011; Willner, 1984). For the behavioral tests utilized in the present dissertation, predictive validity, often argued as most important, is supported by previous validation using a variety of antidepressants (Slattery and Cryan, 2012) and anxiolytics (Cryan and Sweeney, 2011). Evaluation of face validity in these tests, however, has limitations, owing to the fact that fulfillment of this criterion requires the anthropomorphizing of animals (Krishnan and Nestler, 2011). Also, the breadth of symptoms found in the human condition cannot be fully modeled in rodents, as it is difficult if not impossible to assess numerous features of depression and anxiety in non-human animals. For example, depressed mood (e.g. feelings of sadness, emptiness, hopelessness), feelings of worthlessness, excessive or inappropriate guilt, recurrent thought of death, suicidal ideation, suicide attempts, or having a specific plan for committing suicide ("Depressive disorders," 2013) are symptoms that cannot be assessed in rodents. Additionally, symptoms of anxious distress, which include "feeling keyed up or tense" and "difficulty concentrating because of worry" ("Depressive disorders," 2013), are also difficult to investigate in a rodent model. Lastly, construct validity cannot be determined at this time, as this criterion suffers from an inherent flaw in that the causes of depression and anxiety in humans are not fully clarified (Krishnan and Nestler, 2011).

In addition to our behavioral findings, data in this dissertation provide insight into the long-lasting impact of PAE on the neurocircuitry and stress-related receptor systems involved in behavior, emotion and the stress response, which may contribute to the increased risk of developing psychopathologies following PAE. While we were able to localize differential gene expression in specific nuclei and reveal potential neurobiological underpinnings for behavioral changes following PAE and CUS, our results do not indicate changes in protein levels and function. Although findings from a recent study provide support for the implicit assumption that protein levels would correspondingly change with alterations in mRNA expression (Koussounadis et al., 2015), mRNA expression results cannot account for post-translational modifications of proteins which can impact functional outcome by altering activity, stability, amount, localization, and protein-ligand and protein-protein interactions. PAE may exert influences on processes downstream of transcription which can in turn impact behavioral output; therefore, further experimentations are warranted. Nevertheless, our findings can serve as a guide for future experiments (e.g. knock-in, knock-out/down, transgenic, siRNA, optogenetics) that directly examine the relationship between changes in expression of specific genes and behavioral outcome.

To examine the role of HPA hyperresponsivity to stress – in particular, hypersecretion of corticosterone – in mediating the effects of PAE and CUS on brain and behavior, ADXR was utilized to clamp corticosterone at low basal levels. Chronic adrenalectomy has been suggested, however, to induce hippocampal granule cell degeneration (McNeill et al., 1991; Roy et al., 1990; Sloviter et al., 1993, 1989; Spanswick et al., 2011). Given that the hippocampus is

involved with spatial learning and emotional and stress regulation, there may be implications for behavior. Corticosterone replacement at low basal levels may help protect the hippocampus under *non-stressed* conditions from adrenalectomy-induced degeneration (Lee et al., 2011; McCormick et al., 1997). However, it is unknown whether clamping corticosterone at low basal levels may have detrimental consequences on hippocampal granule cells under chronically *stressful* environments, as with the present study, and will require further investigations. Despite the potential effects of ADXR on the hippocampus, given that the behavioral tests used are primarily tests of unconditioned/unlearned behavioral responses, our data on the effects of PAE and CUS on behavioral responsivity to stress remain relevant and meaningful.

5.3 Future directions

Data presented in this dissertation indicate alterations in both central top-down (MR, GR, and CRHR1 expression) and bottom-up (corticosterone-mediated negative feedback) regulation of the HPA axis following PAE and CUS. As noted, there are limitations in examining only mRNA expression, and investigation of other relevant mechanistic aspects of HPA regulation will certainly enrich the present data. An obvious follow-up study would be to examine the impact of PAE on total receptor levels; however, while that may be informative, examining the effects of PAE on localization – nuclear vs cytosolic vs membrane – and splice variants of glucocorticoid receptors may provide a more thorough evaluation. Indeed, it has been reported that while total levels of GR and MR were unchanged in unstressed adolescent PAE mice, nuclear localization of the receptors in the hippocampus and PFC was altered and these impairments were associated with decreased hippocampus-dependent learning and memory and inflexibility in frontal cortical-dependent reversal learning (Allan et al., 2014; Caldwell et al., 2014). Therefore, altered

localization of receptor proteins may have functional implications. Additionally, membrane GR and MR exist that, in response to stress, exert non-genomic effects distinct from cytoplasmic/nuclear receptors (Joëls and de Kloet, 2017; Rainville et al., 2017). Moreover, multiple isoforms of GR and MR can occur due to alternative splicing (Gomez-Sanchez and Gomez-Sanchez, 2014; Merkulov et al., 2017; Vandevyver et al., 2014). Physiological significance of the MR splice variants is uncertain (Gomez-Sanchez and Gomez-Sanchez, 2014); by contrast, the GR splice variants have different functions. For example, GRβ inhibits the activity of GRα ("classic" GR), as well as induces/suppresses a large number of genes that are not regulated by GRα (Merkulov et al., 2017; Oakley and Cidlowski, 2013). Alternative translation initiation also results in translational isoforms of GRα that have different transcriptional activity, and environmental factors (e.g. stressors) can influence the ratio of these isoforms (Merkulov et al., 2017). Of relevance, GRα mRNA and/or protein in the amygdala, cingulate gyrus, and prefrontal cortex are found to be lower in post-mortem samples of depressed patients or suicide victims than healthy controls (Merkulov et al., 2017). As well, GRβ expression can be induced by proinflammatory cytokines, which may play a role in depression (discussed below) (Merkulov et al., 2017; Oakley and Cidlowski, 2013). Taken together, an integrated approach in examining the complex molecular machinery of GR- and MR-mediated regulation of the HPA stress response following PAE will help gain a more complete understanding of the mechanisms that drive increased risk of developing depression and anxiety and may help identify novel targets for effective interventions and treatments.

In addition to effects on HPA regulation, PAE is known to impact other systems that may underlie an increased risk for psychopathologies. Notably, PAE has been found to alter the

development of the neuroimmune system (Bodnar et al., 2016), which may have implications for depression and anxiety, as inflammation has been suggested to play a role in the etiology or disease progression of mental health disorders (Derry et al., 2015; Lucassen et al., 2014; Miller and Raison, 2016; Raison and Miller, 2013). Importantly, the (neuro)immune system interacts with the endocrine stress axis, and both clinical and preclinical studies find that exposure to psychosocial stress, one of the risk factors of depression, can activate an inflammatory response (Derry et al., 2015; Lucassen et al., 2014; Miller and Raison, 2016; Raison and Miller, 2013). Interestingly, antidepressants can alter levels of pro-inflammatory and anti-inflammatory cytokines (Horowitz et al., 2015). Anti-inflammatory agents, however, are effective only in individuals with depression who also demonstrate increased peripheral inflammation, and can have detrimental effects in those without inflammation (Miller and Raison, 2016). Nevertheless, given that PAE may alter sensitivity to chronic stress and modulate the development of the neuroimmune system, future work using a multidisciplinary approach to probe at the effects of PAE on neuroimmune function and HPA regulation will be instrumental in elucidating the underlying mechanisms in the pathophysiology of mental health disorders in FASD and may help develop novel anti-inflammatory-based treatment strategies for affected individuals.

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Appendices

Appendix A Dam and offspring body weights (g, mean ± SEM)

Body weight of dams	Treatment during pregnancy		
	Control	Pair-fed	Alcohol-exposed
Pregnant dams (N)	18	18	21
Maternal death/illness (N)	0	0	0
Perinatal death	0.7 ± 0.2	0.5 ± 0.2	0.3 ± 0.2
Litter size	14.6 ± 0.6	14.1 ± 0.6	14.8 ± 0.6
Total litter mass	132.0 ± 12.2	98.0 ± 11.9	98.4 ± 11.3
Dam weight (g):			
GD 1	289.6 ± 4.0	292.8 ± 4.0	287.4 ± 3.7
GD 7	326.4 ± 4.3	312.3 ± 4.3	305.0 ± 4.0
GD 14	378.8 ± 5.8	353.6 ± 5.8	346.9 ± 5.3
GD 21	478.6 ± 7.9	444.3 ± 7.9	432.1 ± 7.3
LD 1	385.0 ± 7.3	365.1 ± 6.7	344.9 ± 6.4[a]
LD 8	371.4 ± 7.1	361.2 ± 6.6	357.3 ± 6.2
LD 15	372.2 ± 6.5	364.1 ± 6.0	369.4 ± 5.7
LD 22	344.8 ± 5.4	339.6 ± 4.9	346.9 ± 4.7

Body weight of offspring	Prenatal treatment					
	Control		Pair-fed		PAE	
Males						
PND 1	6.9 ± 0.2		7.3 ± 0.2		6.9 ± 0.2	
PND 8	17.3 ± 0.4		18.3 ± 0.4		18.0 ± 0.4	
PND 15	33.3 ± 1.3		35.1 ± 1.2		34.0 ± 1.2	
PND 22	54.4 ± 1.3		56.4 ± 1.3		58.1 ± 1.2	
Pre-surgery (PND 54-64)	351.5 ± 6.4		364.1 ± 6.4		370.5 ± 6.4	
	Sham	*ADXR*	*Sham*	*ADXR*	*Sham*	*ADXR*
Pre-CUS (PND 62-72)	403.4 ± 9.9	397.3 ± 9.6	405.0 ± 9.9	419.2 ± 9.6	442.3 ± 9.9[b]	398.0 ± 9.6[c]
Females						
PND 1	6.9 ± 0.2		6.8 ± 0.2		6.6 ± 0.2	
PND 8	16.7 ± 0.4		17.5 ± 0.4		17.3 ± 0.4	
PND 15	33.7 ± 1.3		33.6 ± 1.3		34.3 ± 1.2	
PND 22	53.1 ± 1.2		54.5 ± 1.2		55.4 ± 1.1	
Pre-surgery (PND 54-64)	222.9 ± 3.1		233.1 ± 3.1[d]		238.1 ± 3.1[d]	
	Sham	*ADXR*	*Sham*	*ADXR*	*Sham*	*ADXR*
Pre-CUS (PND 62-72)	245.9 ± 4.9	238.1 ± 4.9	253.6 ± 4.9	243.0 ± 4.9	256.1 ± 4.9	250.7 ± 4.9

[a] PAE < C; [b] Sham PAE > Sham C = Sham PF; [c] ADXR PAE < Sham PAE; [d] PAE = PF > C

Appendix B Corticosterone intake (mean mg/kg body weight ± SEM)

	MALES			FEMALES		
	C	PF	PAE	C	PF	PAE
n	10	10	9	9	9	10
Pre-CUS	3.03 ± 0.34	3.18 ± 0.20	3.67 ± 0.22	12.26 ± 0.59	11.58 ± 1.11	13.76 ± 0.78

	Non-CUS			CUS			Non-CUS			CUS		
	C	PF	PAE	C	PF	PAE	C	PF	PAE	C	PF	PAE
n	5	5	5	4	5	5	5	5	5	5	5	5
Post-CUS	3.49 ± 0.38	2.88 ± 0.29	3.91 ± 0.38	3.88 ± 0.67	3.13 ± 0.40	3.25 ± 0.09	10.71 ± 2.14	13.02 ± 1.48	14.33 ± 1.13	14.60 ± 0.97	12.95 ± 1.79	15.19 ± 1.47

Appendix C Summary tables of missing samples or extreme outliers in each chapter

	Non-CUS			CUS-1			CUS-14		
Chapter 2	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**
Forced swim test – day 1	-	-	-	-	-	-	1F	-	-
Reason(s):	Extreme outlier								
Forced swim test – day 2	-	-	-	1F	-	-	-	-	1F
Reason(s):	Extreme outliers								
c-fos mRNA – medial prefrontal cortex – all subregions	-	-	-	-	-	1M	-	-	-
Reason(s):	Slides from vendor were contaminated								
c-fos mRNA – amygdala – all nuclei	-	1F	1M	1M	1F	-	-	-	-
Reason(s):	Slides from vendor were contaminated								
c-fos mRNA – hippocampal formation – DG, CA3, CA1	2M; 1F	2F	1M	1M; 1F	1F	2M; 1F	-	-	-
Reason(s):	Slides from vendor were contaminated								
c-fos mRNA – hippocampal formation – ventral subiculum	2M ; 1F	2F	1M	2M; 1F	1F	2M; 1F	-	-	-
Reason(s):	Slides from vendor were contaminated								
c-fos mRNA – hypothalamus – medial parvocellular dorsal division	1M; 1F	1F	-	-	1F	-	-	-	-
Reason(s):	Slides from vendor were contaminated								

M = male; F = female

	Non-CUS			CUS-1			CUS-14		
Chapter 3	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**
Corticosterone – pre-CUS	1M	1M	1M	N/A	N/A	N/A	N/A	N/A	N/A
Reason(s):	Extreme outliers								
Corticosterone – post-CUS	-	-	-	1M	-	-	N/A	N/A	N/A
Reason(s):	Extreme outlier								
Open field	-	-	-	-	-	-	-	-	-
Reason(s):	N/A								
Dark-light emergence	1F[a]				1M[b]				2M[c]
Reason(s):	[a] Animal did not perform the task (no behavior other than 1 nose poke) [b] Extreme outlier [c] 1 animal did not perform the task (no behavior other than 1 nose poke); 1 extreme outlier								
Elevated plus maze	-	-	-	-	-	-	-	-	-
Reason(s):	N/A								
CRHR1 mRNA – medial prefrontal cortex – all subregions	1M; 1F	1F	1M	1M; 1F	-	1M	-	-	-
Reason(s):	Slides from vendor were contaminated								
CRHR1 mRNA – amygdala – all nuclei	-	2F	1M	1M	1M	-	-	1M	-
Reason(s):	Slides from vendor were contaminated								
CRHR1 mRNA – hippocampal formation – all subregions	1M	2F	1M	1M	1F	1F	1F	1M	1F
Reason(s):	Slides from vendor were contaminated								
MR mRNA – hippocampal formation – all subregions	-	-	-	-	1F	-	-	-	-
Reason(s):	Slides from vendor were contaminated								
GR mRNA – medial prefrontal cortex – all subregions	1M; 1F	2F	1M	1M; 1F	-	1F	-	-	-
Reason(s):	Slides from vendor were contaminated								
GR mRNA – amygdala – all nuclei	1M	2F	1M	1M; 1F	-	-	-	1F	-
Reason(s):	Slides from vendor were contaminated								
GR mRNA – hippocampal formation – all subregions	2M	1F	1M	2M; 1F	1F	-	-	-	-
Reason(s):	Slides from vendor were contaminated								

M = male; F = female

	Sham						ADXR					
	Non-CUS			CUS			Non-CUS			CUS		
Chapter 4	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**	**C**	**PF**	**PAE**
Basal corticosterone – pre-CUS	3M	2M; 1F	2M; 1F	N/A	N/A	N/A	1M; 1F	3F	1M	N/A	N/A	N/A
Reason(s):	Extreme outliers											
Basal corticosterone – post-CUS	1M[a]	1M[a]; 1F[b]	-	-	-	-	-	-	-	1M[a]	1F[a]	-
Reason(s):	[a] Extreme outliers [b] Not enough sample could be collected											
Basal ACTH – pre-CUS	3F	2M; 2F	1M	N/A	N/A	N/A	-	2F	1M; 1F	N/A	N/A	N/A
Reason(s):	Not enough samples could be collected											
Basal ACTH – post-CUS	-	1M[a]; 2F[b]	-	-	2M[b]; 2F[a]	1F[d]	-	1F[a]	3M[c]; 1F[a]	4F[a]	3M[a]; 3F[a]	2M[a]; 2F[a]
Reason(s):	[a] Not enough samples could be collected [b] Not enough samples could be collected from 1 of the animals; 1 extreme outlier [c] Not enough samples could be collected from 2 of the animals; 1 extreme outlier [d] Extreme outlier											
Open field	-	-	-	-	1M	-	-	-	-	-	-	-
Reason(s):	Lost data due to equipment failure											
Elevated plus maze – percent of time in the open arms only	1F	-	-	-	-	-	-	-	1M	-	-	2M
Reason(s):	Extreme outliers											
GR mRNA – medial prefrontal cortex – all subregions	-	-	-	-	-	-	1M	-	-	-	-	-
Reason(s):	Tissue damaged during processing											
GR mRNA – amygdala – all nuclei	-	-	-	-	-	-	1M	-	-	-	-	1M
Reason(s):	Tissue damaged during processing											
GR mRNA – hippocampal formation – ventral subiculum only	-	-	-	-	-	-	-	-	1M	-	-	-
Reason(s):	Extreme outlier											
MR mRNA – hippocampal formation – ventral subiculum only	1M	-	-	-	-	-	-	-	-	-	-	-
Reason(s):	Extreme outlier											

M = male; F = female

Appendix D Summary table of significant interactions or main effects of sex

Chapter 2	**Effect**	**F**	***p***
Body weight pre-CUS	Main effect of sex	$F_{1,138} = 977.091$	<0.001
Change in body weight post-CUS	Sex x CUS interaction Main effect of sex	$F_{1,132} = 16.746$ $F_{1,132} = 115.064$	<0.001 <0.001
Forced swim test – immobility – day 1	Sex x CUS interaction Main effect of sex	$F_{1,125} = 6.246$ $F_{2,125} = 17.021$	0.003 <0.001
Forced swim test – swimming – day 1	Sex x CUS interaction Main effect of sex	$F_{1,125} = 7.489$ $F_{2,125} = 24.216$	0.001 <0.001
Forced swim test – climbing – day 1	-	-	-
Forced swim test – immobility – day 2	Sex x CUS interaction Main effect of sex	$F_{1,124} = 87.307$ $F_{2,124} = 15.204$	<0.001 <0.001
Forced swim test – swimming – day 2	Sex x CUS interaction Main effect of sex	$F_{1,124} = 73.008$ $F_{2,124} = 11.738$	<0.001 <0.001
Forced swim test – climbing – day 2	Sex x CUS interaction Main effect of sex	$F_{1,124} = 16.213$ $F_{2,124} = 4.748$	<0.001 0.010
c-fos mRNA – medial prefrontal cortex – Cg1	Main effect of sex	$F_{1,90} = 17.276$	<0.001
c-fos mRNA – medial prefrontal cortex – prelimbic	Main effect of sex	$F_{1,90} = 25.851$	<0.001
c-fos mRNA – medial prefrontal cortex – infralimbic	-	-	-
c-fos mRNA – amygdala – lateral nucleus	-	-	-
c-fos mRNA – amygdala – basal nucleus	-	-	-
c-fos mRNA – amygdala – central nucleus	-	-	-
c-fos mRNA – amygdala – medial nucleus	-	-	-
c-fos mRNA – hippocampal formation – dentate gyrus	-	-	-
c-fos mRNA – hippocampal formation – CA3	-	-	-
c-fos mRNA – hippocampal formation – CA1	-	-	-
c-fos mRNA – hippocampal formation – ventral subiculum	-	-	-
c-fos mRNA – hypothalamus – medial parvocellular dorsal division	Main effect of sex	$F_{1,87} = 8.445$	0.005

Chapter 3	**Effect**	**F**	***p***
Corticosterone – pre-CUS	Main effect of sex	$F_{1,135} = 47.791$	<0.001
Corticosterone – post-CUS	Main effect of sex	$F_{1,130} = 18.848$	<0.001
Open field – center entries	-	-	-
Open field – time in center	-	-	-
Open field – latency to center	Main effect of sex	$F_{1,126} = 8.608$	0.004
Open field – distance (center)	-	-	-
Open field – distance (total)	-	-	-
Dark-light emergence – latency to emerge	Main effect of sex	$F_{1,122} = 51.441$	<0.001
Dark-light emergence – time in light	Main effect of sex	$F_{1,122} = 52.250$	<0.001
Elevated plus maze – percent of time in the open arms	Main effect of sex	$F_{1,126} = 12.608$	0.001
Elevated plus maze – frequency of closed arm entries	Main effect of sex	$F_{1,126} = 8.988$	0.003
CRHR1 mRNA – medial prefrontal cortex – Cg1	-	-	-
CRHR1 mRNA – medial prefrontal cortex – prelimbic	-	-	-
CRHR1 mRNA – medial prefrontal cortex – infralimbic	-	-	-
CRHR1 mRNA – amygdala – lateral nucleus	-	-	-
CRHR1 mRNA – amygdala – basal nucleus	-	-	-
CRHR1 mRNA – amygdala – medial nucleus	Main effect of sex	$F_{1,84} = 4.629$	0.034
CRHR1 mRNA – hippocampal formation – CA3	-	-	-
CRHR1 mRNA – hippocampal formation – CA1	-	-	-
CRHR1 mRNA – hippocampal formation – ventral subiculum	-	-	-
MR mRNA – hippocampal formation – dentate gyrus	-	-	-
MR mRNA – hippocampal formation – CA3	-	-	-
MR mRNA – hippocampal formation – CA1	-	-	-
MR mRNA – hippocampal formation – ventral subiculum	-	-	-
GR mRNA – medial prefrontal cortex – Cg1	-	-	-
GR mRNA – medial prefrontal cortex – prelimbic	-	-	-
GR mRNA – medial prefrontal cortex – infralimbic	-	-	-
GR mRNA – amygdala – lateral nucleus	-	-	-
GR mRNA – amygdala – basal nucleus	-	-	-
GR mRNA – amygdala – central nucleus	-	-	-
GR mRNA – amygdala – medial nucleus	Main effect of sex	$F_{1,83} = 9.492$	0.003
GR mRNA – hippocampal formation – dentate gyrus	Main effect of sex	$F_{1,82} = 6.294$	0.014
GR mRNA – hippocampal formation – CA3	-	-	-
GR mRNA – hippocampal formation – CA1	-	-	-
GR mRNA – hippocampal formation – ventral subiculum	-	-	-

Chapter 4	**Effect**	**F**	***p***
Change in body weight	Group x Sex x CUS interaction Group x Sex Surgery interaction Sex x CUS interaction Main effect of sex	$F_{2,219} = 3.099$ $F_{2,219} = 3.361$ $F_{1,219} = 20.205$ $F_{1,219} = 537.595$	0.047 0.036 <0.001 <0.001
Basal corticosterone – pre-CUS	Sex x surgery interaction Main effect of sex	$F_{1,217} = 14.035$ $F_{1,217} = 28.678$	<0.001 <0.001
Basal corticosterone – post-CUS	Sex x CUS interaction Sex x surgery interaction Main effect of sex	$F_{1,214} = 7.614$ $F_{1,214} = 24.449$ $F_{1,214} = 60.013$	0.006 <0.001 <0.001
Basal ACTH – pre-CUS	Sex x surgery interaction Main effect of sex	$F_{1,218} = 7.210$ $F_{1,218} = 13.044$	0.008 <0.001
Basal ACTH – post-CUS	Main effect of sex	$F_{1,192} = 7.482$	0.007
Open field – time in center	Sex x group x CUS interaction Main effect of sex	$F_{2,218} = 5.269$ $F_{1,218} = 7.843$	0.006 0.006
Open field – center entries	Sex x group x surgery interaction Sex x group x CUS interaction Main effect of sex	$F_{2,218} = 3.771$ $F_{2,218} = 3.358$ $F_{1,218} = 15.523$	0.025 0.037 <0.001
Open field – distance in center	Sex x group x CUS interaction Main effect of sex	$F_{2,218} = 3.169$ $F_{1,218} = 14.730$	0.044 <0.001
Open field – distance (total)	Main effect of sex	$F_{1,218} = 24.056$	<0.001
Elevated plus maze – percent of time in the open arms	-	-	-
Elevated plus maze – closed arm entries	Main effect of sex	$F_{1,219} = 23.179$	<0.001
GR mRNA – medial prefrontal cortex – Cg1	-	-	-
GR mRNA – medial prefrontal cortex – prelimbic	Sex x group x CUS x surgery interaction	$F_{2,140} = 3.503$	0.033
GR mRNA – medial prefrontal cortex – infralimbic	Sex x group x CUS x surgery interaction	$F_{2,140} = 5.192$	0.007
GR mRNA – amygdala – lateral nucleus	-	-	-
GR mRNA – amygdala – basal nucleus	-	-	-
GR mRNA – amygdala – central nucleus	-	-	-
GR mRNA – amygdala – medial nucleus	-	-	-
GR mRNA – hippocampal formation – dentate gyrus	-	-	-
GR mRNA – hippocampal formation – CA3	Sex x surgery interaction	$F_{1,141} = 5.052$	0.026
GR mRNA – hippocampal formation – CA1	-	-	-
GR mRNA – hippocampal formation – ventral subiculum	-	-	-
MR mRNA – hippocampal formation – dentate gyrus	Sex x surgery interaction Main effect of sex	$F_{1,141} = 6.044$ $F_{1,141} = 6.632$	0.015 0.011
MR mRNA – hippocampal formation – CA3	-	-	-
MR mRNA – hippocampal formation – CA1	-	-	-
MR mRNA – hippocampal formation – ventral subiculum	-	-	-