SEX DIFFERENCES IN HYPOTHALAMIC-PITUITARY-ADRENOCORTICAL AXIS
HORMONES AND STRESS-RELATED NEUROCIRCUITRY FOLLOWING
ACUTE AND REPEATED STRESS IN RATS

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ABSTRACT

Stress can be a causative or exacerbating factor for many physical and psychiatric disorders. Several stress-related mood and anxiety disorders are about twice as prevalent in women as in men, such as major depressive disorder, generalized anxiety disorder, and posttraumatic stress disorder. Dysfunction and/or dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis have been implicated in each one of these psychopathologies. Therefore understanding how sex can affect the biological function of the HPA axis may inform the mechanism behind a female’s vulnerability or susceptibility to the maladaptive effects of stress. The studies presented in this dissertation investigate the nature of sex differences in the HPA axis, and how the effect of sex on HPA axis function differs in a stress-specific way in rats. Specific focus is placed on extra-hypothalamic stress responsive neurocircuity, and these chapters provide evidence that when sex differences in acute stress-induced HPA axis hormone release are observed, parallel differences are observed in the activation of several key brain regions known to modulate HPA axis function. Conversely, when no effect of sex on HPA axis hormone release is observed in response to a stressor of a different modality, brain activation of stress responsive neurocircuity is also comparable in the male and female brain. Furthermore, regardless of the effect of sex on acute stress-
induced HPA axis activity, repeated presentations of the same stressor produced similar extent of adaptation (habituation) of HPA axis activity. These data suggest that responses of the HPA axis to relatively mild stressors, whether acutely or repeatedly presented, are not likely to produce maladaptive changes in the female brain that could explain vulnerability to certain stress-related mental illnesses in women. Further research is needed to understand what could produce maladaptive changes to stress specifically in women.
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CHAPTER ONE: GENERAL INTRODUCTION

An organism’s stress response.................................................................2

Relationship of stress to physical and mental health in humans.........................5

Effect of sex on human stress responses and health........................................8

The connection between reproductive hormones and stress responses............12

Effect of sex on stress responses in animals................................................15

The effect of sex and sex steroids on central stress neurocircuitry....................17

Thesis overview......................................................................................19
LIST OF TABLES

CHAPTER FOUR

Table 4.1. The effect of sex on adrenal and thymus weight following repeated stress.........................................................................................................................116
LIST OF FIGURES

CHAPTER TWO

Figure 2.1. Effect of sex and estrous cycle on HPA axis hormone concentrations immediately prior to and following 30 min of restraint stress in Experiment 1……39

Figure 2.2. Plasma estradiol concentrations and relative c-fos mRNA expression in the BSTav, MPOA, and PVN from Experiment 1……………………………………..43

Figure 2.3. Representative photomicrographs displaying the effect of sex on c-fos mRNA expression within the BSTav, MPOA, and PVN of female and male brains from Experiment 1………………………………………………………44

Figure 2.4. Representative photomicrographs of c-fos and CRF mRNA labeled neurons using dual FISH within the BSTav, MPOA, and PVN of female and male brains from Experiment 1………………………………………………………46

Figure 2.5. Quantification of the effect of sex on cells containing CRF, c-fos mRNA, and double-labeled cells as visualized by FISH in Experiment 1 within the BSTav, MPOA, and PVN………………………………………………………47

Figure 2.6. Effect of ovariectomy with or without estradiol replacement compared to intact male and female rats on plasma hormone concentrations following restraint stress from Experiment 2…………………………………50

CHAPTER THREE

Figure 3.1. The effect of sex on ACTH and CORT concentrations at baseline (60 dB) and after 30 min of low-intensity noise stress (75 and 80 dB)…………………80

Figure 3.2. The effect of sex on the recovery from HPA activation in response to 30 min of 90 dB noise……………………………………………………………………82

Figure 3.3. The effect of sex on relative c-fos mRNA expression at baseline and following 30 min of 90 dB noise stress in stress-related and auditory processing brain regions…………………………………………………………83

Figure 3.4. The effect of sex on pre-pulse suppression (GAP and PPI) and facilitation (PPF) of the acoustic startle response…………………………………84

Figure 3.5. The effects of sex and ovariectomy with or without estradiol replacement on HPA axis responses to 30 min of 105 dB noise…………………86
Figure 3.6. The stressor specific effect of sex on HPA axis responses in response to 30 min of restraint, 80 dB, and 105 dB noise stress in the same male and female rats in proestrus .................................................................88

Figure 3.7. The effects of sex and social context of stress on CORT concentrations over time .............................................................90

Figure 3.8. The effect of sex on HPA axis responses to 30 minutes of restraint without experience of a basal blood sample.............................................92

CHAPTER FOUR

Figure 4.1. The effect of sex, handling, and repeated stress on body weight gain across Days 1-10 ...........................................................................110

Figure 4.2. The effect of sex HPA axis hormone concentrations in response to 10 days of repeated noise stress .........................................................112

Figure 4.3. The effect of sex HPA axis hormone concentrations in response to 10 days of repeated restraint stress .....................................................114

Figure 4.4. The effect of sex and prior repeated noise or restraint stress on HPA axis hormone release following exposure to a novel environment .............118
CHAPTER ONE:

GENERAL INTRODUCTION
An organism’s stress response

There are a multitude of situations that can prove harmful to an organism. Being chased by a predator, lack of nutrition, extreme temperature fluctuations, and infection are some examples from the broad category of physically harmful situations. Other situations that have the potential to lead to a physically harmful situation, such as predator cues (like odor), a confined space, or social conflict can be perceived as threatening to an organism’s safety, even if the situation does not result in immediate harm. Luckily, there are also evolutionarily conserved ways of increasing chances for survival in many of these harmful situations. These survival mechanisms are considered to be highly adaptive, functioning to maintain an internal balance, a physiologically dynamic equilibrium coined homeostasis (Cannon, 1932). The term stress, coined by Hans Selye approximately half a century ago (Selye, 1956), refers not only to the situation that is perceived as actually or potentially threatening (a stressor), but also to the changes it produces in the organism with the purpose of maintaining homeostasis (stress response), and finally to the behavior of the organism, meant to optimize survival, that the stressor and stress response created (Levine and Ursin, 1991). Upon first glance, it may appear that there is no system in, or part of, the body that can be considered to be unresponsive to stress stimuli. However, there are a few key systems that modulate intricate and often elaborate physiological responses to stress.

Two of the main physiological systems involved in stress responses are the hypothalamic-pituitary-adrenocortical (HPA) axis, and the sympatoadrenomedullary division of the autonomic nervous system (Chrousos & Gold, 1992; Stratakis &
Chrousos, 1995). The latter system mostly controls the main acute “fight-or-flight” homeostatic responses to stress, such as dilation of pupils, blood glucose increases, and changes in respiration, heart rate, and blood pressure with peripheral release of catecholamines from the adrenal medulla (Chrousos & Gold, 1992). The HPA axis on the other hand, when activated, mostly contributes to the immediate survival of the organism by stimulating the production of glucocorticoids (GC). This is accomplished by parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN), that when activated, stimulate the production and release of corticotropin releasing factor (CRF) and arginine vasopression (AVP) into the hypophyseal portal blood system. CRF and AVP then travel a short distance through this highly specific blood supply to the anterior pituitary, which stimulates corticotroph cells to produce and release adrenocorticotropic hormone (ACTH) into the general blood supply. ACTH then travels via the organism’s circulatory system where it stimulates the adrenal cortex to produce and release GCs into the blood. The GCs cortisol (the main GC in humans) and corticosterone (the main GC in rodents; CORT) main function to promote survival is to induce gluconeogenesis, which allows large amounts of energy to be available for successful completion of fight or flight behaviors. GCs also serve as their own negative feedback modulators, and can inhibit further production of GCs at the level of the PVN, pituitary, and adrenal glands (direct negative feedback), but also largely at the level of the hippocampus (Herman et al, 1989) and potentially other extra-hypothalamic brain regions (indirect negative feedback).

In addition to reciprocal interconnections coordinating an adaptive stress response between the HPA axis and the sympathoadrenal and other stress-induced
systems (Brown et al, 1985; Sawchenko & Swanson, 1985, Chrousos & Gold, 1992; Kvetnansky et al, 1995), many other functions that are not essential to an organism’s immediate survival are influenced by the HPA axis in stressful situations. For example, the HPA axis can affect reproductive, thyroid, and growth systems, as well as gastrointestinal, metabolic, and immune function (Stratakis & Chrousos, 1995; McEwen et al 1997; Nelson 2005). It is mostly through the actions of the central effector of the HPA axis, the hormone/neuropeptide CRF, that HPA axis stress-induced inhibition on many other systems of the body (e.g., growth, digestive, and reproductive function) is accomplished, and this is further evidenced by the presence of CRF-producing cells in peripheral immune and reproductive systems (Tsigos & Chrousos, 2002; Kalantaridou et al, 2007). Therefore the HPA axis also contributes indirectly to survival of an organism experiencing stress by redirecting energy toward the immediate, or emergency, needs of that organism.

In addition to these two main peripheral systems just described, multiple systems are also activated within the central nervous system (CNS). For example, the locus coeruleus (LC) in the brainstem controls the release of the catecholamine norepinephrine (NE) into many regions within the brain. Release of NE from the LC can be thought of as the main modulator of an organism’s state of arousal (Aston-Jones et al, 1991; Koob 1999; Morilak et al, 2005), and is thought to control stress-induced enhanced arousal, hypervigilance, and possibly anxiety (Chrousos & Gold, 1992; Morilak et al, 2005). Another important CNS modulator of stress-induced cognitive states is CRF, which is produced not only in the PVN, but also limbic structures, such as the amygdala and bed nucleus of the stria terminalis (BST), several regions of the
cortex, and brainstem structures (Claes 2004). In particular, there is extensive evidence that two of these limbic structures, specifically, the central nucleus of the amygdala (CeA) and the lateral BST, are associated with fear and anxiety states (Campeau et al, 1991; Campeau et al, 1992; Davis et al, 1993; Campeau & Davis, 1995; Davis et al, 1997a; Davis et al, 1997b; Davis 1998; Walker et al, 2003; Day et al 2005; Schulkin et al, 2005; Toufexis 2007; Day et al, 2008; Walker et al, 2009; Spannuth et al 2011; Bienkowski & Rinaman, 2012). It has been suggested that it is a GC-independent (and thus independent of HPA axis activation) CRF system that is important in the general regulation of fear and anxiety states mediated by these two limbic structures (Liang et al 1992a; 1992b; Schulkin et al, 1998; Deak et al, 1999). For example, one type of psychological stress, which is defined as a situation that activates an organism’s stress responses and is not physically harmful, but not a physiological stressor, significantly increased CRF gene (mRNA) expression in the CeA, an effect that was NOT also observed in the PVN (Makino et al, 1999). Another psychological stressor, the odor but not presence of a predator, has been shown to significantly activated the BST, but not the PVN (Figueiredo et al, 2003). Thus it is evident that CRF is important for both HPA axis responses as well as affective states associated with experiencing stress.

Relationship of stress to physical and mental health in humans

Although stress responses have evolved to aid survival in many potentially harmful situations, excessive or prolonged responses to stress are generally maladaptive. One striking example of the maladaptive effects of chronic HPA axis activity comes from patients with Cushing’s disease, in which a tumor, originating either in the pituitary or adrenal gland, produces supraphysiological and sustained high levels
of GCs in the body. Patients with this disease often have multifaceted physiological problems, as one might suspect given the adaptive interactions of the HPA system with other systems of the body, including hypertension, weight gain and/or obesity, protein and/or muscle wasting, hirsutism (excessive body hair), reduced fertility, osteoporosis, and metabolic symptoms such as insulin resistance leading to hyperglycemia and diabetes (Castinetti et al, 2012). This disease can also be mimicked by exogenous treatment with GCs. However, Cushing’s disease (whether endogenous or exogenous) is not the only situation that can produce excessive amounts of GCs. Many other physical health problems have been associated with dysfunction of the HPA axis, both in situations of increased HPA axis activity, which has been associated with hyperthyroidism, diabetes and cardiovascular disease (Wolff 1950), and in situations of decreased HPA axis activity, which has been associated with chronic fatigue syndrome, fibromyalgia, hypothyroidism, and rheumatoid arthritis (Stratakis & Chrousos, 1995). Thus, the aforementioned negative feedback system of HPA axis that carefully regulates the amount of GCs within the blood is a critical defense system in order to prevent the negative consequences of excessive GC.

Importantly, in addition to the plethora of physically potentially harmful situations mentioned above, stress systems, including the HPA axis, can be activated by situations that are difficult to categorize as immediately threatening to an organism's survival. Seemingly innocuous situations relevant to everyday human life such as negotiating rush hour traffic, taking an exam or being evaluated at work, and public speaking also have the potential to be potent activators of stress responses. The fact that psychological stressors such as these, which are ubiquitous to nearly all humans in
the developed world, can be perceived as stressful is not trivial. Experiencing psychological stressors as commonplace as the aforementioned daily life hassles, as well as more severe major life events such as the death of a loved one, financial hardship, divorce, and physical trauma have all been linked to both physical and mental health. Stressful life events have been linked to the onset and severity of depression (Dunner et al, 1979; Brown et al, 1986; 1987; Hammen et al 1992; Claes 2004; Young & Altemus, 2004), and events such as these occurring in early life may render one particularly susceptible to psychiatric illness (Heim et al, 1997a; 1997b; McEwen 2000; Romeo 2010). Reciprocally, psychological wellbeing can also profoundly affect physical health outcomes. Diagnosis of major depressive disorder (MDD), as defined by the Diagnostic and Statistical Manual of mental disorders, 4th edition (DSM-IV), has been linked to osteoporosis and cardiovascular illness (Claes 2004), and depressive symptoms are associated with failure to complete cardiac rehabilitation in patients with coronary heart disease (Caulin-Glaser et al, 2007). Because of these relationships to somatic illness, depression has also been linked with increased morbidity and mortality by the age of 70, even when other factors were carefully controlled for, such as diet and smoking (Vaillant 1998).

Extensive research has been aimed at attempting to identify the underlying biological mechanisms for the detrimental psychological stress-induced effects on mental health, and many studies have pointed to maladaptive changes in stress response systems. Dysfunction of the HPA axis, for example, has been linked to several stress-related psychopathologies. Paradoxically, hyperactivity of the HPA axis is associated with melancholic depression, alcoholism, obsessive-compulsive and
eating disorders, whereas hypoactivity of the HPA axis is associated with atypical depression, chronic fatigue syndrome, PTSD, and chronic neuropathic pain (Altemus et al, 1992; Stratakis & Chrousos, 1995; Wong et al, 2000; Ehlert et al, 2001; Gold & Chrousos, 2002; Ulrich-Lai et al, 2006). In the case of PTSD however, it is also noted that maladaptive changes in the central regulation of serotonin, NE, and EPI are likely to be especially relevant to PTSD symptoms such as hypervigilance, impulsivity, and aggression (Southwick et al, 1999). Specifically within the HPA axis, it has been suggested that maladaptive function of CRH plays a role in the pathophysiology of both mood and anxiety disorders (Behan et al, 1996; Arborelius et al, 1999). Patients with obsessive-compulsive disorder, for example, indeed have significantly elevated CRH, as well as AVP, in the cerebrospinal fluid compared to controls (Altemus et al, 1992). CRH has even recently been implicated in the comorbidity of depression and cardiovascular disease (Wood et al, 2012). As a result of these findings, drugs targeting the CRH system are now being utilized as treatments for both mood and anxiety disorders (Tsigos & Chrousos, 2002; Hauger et al, 2006; Zoumakis et al, 2006; Hauger et al, 2009; Konstantinos et al, 2009; Lloyd & Nemeroff, 2011).

**Effect of sex on human stress responses and health**

One’s biological sex appears to have a profound effect on stress and stress responses and subsequent negative health consequences. The female immune system appears to be more reactive to stress, leading to men being more susceptible to infection than women, and women being more prone to several autoimmune diseases (Bourke et al, 2012). In addition, men appear to be more susceptible to stress-induced metabolic dysfunction, and are more likely to develop stress-induced metabolic
consequences such as hyperglycemia, hyperinsulinemia, coronary heart disease, and metabolic syndrome (Bourke et al, 2012). Men also display higher prevalence of personality disorders (Hensley & Nurnberg, 2002), as well as alcohol and other substance use disorders compared to women, except in the case of women with prior history of sexual abuse (Canterbury, 2002).

It is when the prevalence of affective psychiatric disorders is considered that a woman’s vulnerability to the effects of stress is uncovered. Women have a higher overall prevalence of developing such affective disorders as depression (Kornstein and Wojcik, 2002), Bipolar II disorder (characterized by episodes of hypomania and depression; Freeman et al, 2002), panic disorder, generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), obsessive-compulsive disorder, agoraphobia, and specific phobia (Pigott 2002). In addition, women are more likely to have an eating disorder (Powers 2002). Women are about three times more likely to develop MDD after experiencing stressful life events, and intriguingly this may be specifically due to increased risk in women experiencing more distant or distal interpersonal life events such as the death of a close friend or other relative, as sex does not affect the risk of developing depression following more proximal interpersonal life events such as the death of a spouse or child, divorce, acute financial or legal trouble (Maciejewski et al, 2001). Once diagnosed, women also exhibit a higher risk of recurring episodes of depression following stressful life events compared to men (Sherrill et al, 1997). Furthermore, depression is highly comorbid with anxiety disorders, especially in women (Kendler 1996). And in fact, depressed patients with comorbid anxiety disorders show more exaggerated stress-induced HPA axis activity compared to depressed patients.
without comorbid anxiety (Young et al, 2004). Women are also more likely to develop PTSD after experiencing a traumatic event (Stein et al, 2000), and women diagnosed with PTSD are also twice as likely to have prolonged PTSD and a worse quality of life compared to men also diagnosed with PTSD following a traumatic event (Holbrook et al, 2002). In general, women appear to be more reactive to stress than men. Women display larger amplitude startle reflexes to aversive (unpleasant) emotional stimuli, and larger positivity of slow evoked potentials to unpleasant compared to pleasant stimuli relative to men in the area of the prefrontal region of the brain as measured by electroencephalogram (EEG), and these results are proposed to, at least partially, explain the female-biased susceptibility to affective psychopathologies (Bianchin & Angrilli, 2012). However, despite the fact that mood and anxiety disorders are the most common psychiatric illnesses, and are clearly sex-biased, one’s biological sex is typically not considered when treatment is decided (Bekker & van Mens-Verhulst, 2007). More research is needed to understand the mechanisms that underlie sex differences in susceptibility to mental illnesses in general before treatments specific for men and women are likely to emerge.

Extensive research has also been aimed at understanding potential physiological bases and/or mechanisms behind female vulnerability to affective psychiatric illness in women, and much attention has been paid to the HPA axis specifically, due to its obvious involvement in mood and anxiety disorders in men and women. In a laboratory setting, there are two main methods used to examine HPA axis function in humans. The dexamethasone suppression test (DST) and the combined dexamethasone suppression/CRH stimulation (DEX/CRH) test measure the sensitivity of the HPA axis
by examining the ability of the synthetic glucocorticoid DEX to suppress endogenous GC levels (DST), combined with the ability of CRH to then stimulate ACTH and GC release (DEX/CRH test; Heuser et al, 1994b; Kunugi et al, 2006). Escape from GC suppression in response to dexamethasone is common in depressed patients, and women are more likely to exhibit this failure to the DST by itself, especially with advancing age (Akil et al, 1993; Deuster et al, 1998). And in both normal control and patients with depression, women also display exaggerated hormonal responses to the combined DEX/CRH test (Heuser et al, 1994a; Kunzel et al, 2003; Kunugi et al, 2006).

In contrast, patients with PTSD and healthy subjects exposed to trauma are reported to show enhanced suppression of cortisol levels following the DST (de Kloet et al, 2006; Jones and Moller, 2011; Klaassens et al, 2012).

The other main method for examining HPA axis function in humans is to administer a psychological stressor called the Trier Social Stress Test (TSST), which generally consists of mental arithmetic tasks and a speech, both performed in front of an audience (Foley & Kirschbaum, 2010). The TSST is a potent activator of stress responses in humans, as evidenced by significantly increased plasma ACTH, plasma and salivary cortisol, blood pressure, and heart rate (Kirschbaum et al, 1999; Kudielka et al, 1999; Singh et al, 1999). In healthy men and women, males consistently display significantly higher ACTH and cortisol responses basally and in response to the TSST (Kirschbaum et al, 1992; Kudielka et al, 1998; Kirschbaum et al, 1999; Uhart et al, 2006; Shalev et al, 2009; Paris et al, 2010), although the experience of early life trauma eliminates the effect of sex on TSST-induced cortisol increases in a healthy population (DeSantis et al, 2011). Also, women who experience early life trauma show increased
GC response to administration of CRH than men (DeSantis et al., 2011). However, in depressed patients, women reportedly exhibit higher basal HPA axis activity compared to male patients with depression (Young et al., 2004). In addition, there is some evidence that sex differences in HPA axis responsiveness to the TSST might be genetically driven, specifically due to sex differences in the genes encoding the glucocorticoid receptor (GR; Kumsta et al., 2007). And interestingly, brain damage, specifically in the medial prefrontal cortex (mPFC), has differential effects in men and women. Specifically, mPFC damage, but not damage in other areas of the brain, significantly increases cortisol responses to the TSST in women, but has the opposite effect in men (Buchanan et al., 2010). In summary, both of these laboratory tests point to biological sex differences in stress system responsiveness, specifically within the HPA axis.

**The connection between reproductive hormones and stress responses**

The existence of psychiatric illnesses specific to women during particular stages of the hormonal and reproductive cycle, such as premenstrual dysphoric disorder (PMDD), postpartum depression (PPD), and postpartum psychosis clearly point to a potential relationship between circulating endogenous sex hormones such as estrogen and progesterone and stress responses. Furthermore, just as common as PPD (that coincides with parturition and a concurrent sudden and dramatic reduction in cortisol) but less well known, depression can occur during pregnancy (when there are not only large increases in plasma estrogen and progesterone, but also enormous increases in plasma CRH and cortisol that can reach levels found in Cushing’s syndrome), and these two types of depression have been hypothesized to mimic atypical and melancholic
forms of depression, respectively, due to these markers of HPA axis activity (Kammerer et al, 2006). However, reproduction per se is not a modifier of the HPA axis, since parity by itself does not appear to alter basal or stress-induced HPA axis activity (Federenko et al, 2006). The menstrual cycle can also affect HPA axis functioning, further pointing to the idea that fluctuating sex steroid hormones can affect stress responsiveness. For example, healthy women display enhanced suppression of cortisol to the DEX suppression test in the follicular compared to the mid-luteal phase of the menstrual cycle (Altemus et al, 1997), and display higher stress-induced AVP and ACTH responses in the midluteal phase (Altemus et al, 2001), supporting the idea that sex steroids (elevated in the luteal phase) have a stimulatory effect on the HPA axis, and make this system resistant to suppression. And finally, some evidence exists that women in the luteal phase have similar HPA axis responses to the TSST, and it is only women in the follicular phase that show reduced responses to this stressor compared to men (Kirschbaum et al, 1999).

Rodent studies focusing on the effect of reproductive hormones generally observe the same phenomena as in humans. The generally accepted current hypothesis is that estrogens enhance, and androgens inhibit, HPA axis responses. For example, exogenous application of estradiol in ovariectomized female rats enhances CORT responses to the DST (Figueiredo et al, 2007), similar to the effect of the DST observed in the luteal phase of the menstrual cycle (Altemus et al, 1997). Further evidence for activational, as opposed to organizational, effects of gonadal steroids on HPA axis function comes from analysis of plasma ACTH and CORT concentrations in response to the DST in intact as compared to gonadectomized male and female rats.
Whereas gonadectomy in male rats enhanced DST suppression of both ACTH and CORT, the opposite effect is observed in ovariectomized female rats (Almeida et al, 1997), suggesting that the presence of gonadal steroids in both sexes exerts effects on the sensitivity of the HPA axis. It is also clear, however, that gonadal steroids also play a very important role in the neonatal organization of the HPA axis, as profound changes in CRH gene expression, brain glucocorticoid receptors, and diurnal CORT are observed if female rat pups are masculinized with estrogen, or male rats are ‘feminized’ with orchidectomy, neonatally (Patchev et al, 1995; 1999). In addition, Weiser and colleagues (2009) demonstrated that the estrogen receptor alpha (ERα) agonist propylpyrazoletriol (PPT) in ovariectomized females increased stress-induced ACTH and CORT levels compared to control female rats, while the ER beta (ERβ) agonist diarylpropionitrile (DPN) decreased these same measures following stress compared to controls. Furthermore, PPT treatment led to reduced inhibition of ACTH and CORT by DEX, whereas DPN had no effect on this measure, suggesting that the differential activation of particular ERs can determine the effect of estradiol on HPA axis in vivo (Weiser & Handa, 2009). In rhesus monkeys, simulation of the follicular phase of the menstrual cycle increased CRF mRNA in the PVN of OVX females, but simulation of the luteal phase produced no change from controls (Roy et al 1999). Indeed, the human CRF gene contains estrogen response elements in the promoter region, supporting the idea that circulating sex steroids can directly affect HPA axis activation (Vamvakopoulos & Chrousos, 1993). Reports have suggested that estrous cycle stage influences on HPA axis responses to acute stress are due to the augmenting effect of estrogen specifically on this system (Viau & Meaney 1991; McCormick et al, 2002; Lunga &
Herbert, 2004; Viau et al, 2004; Figueiredo et al, 2007; Larkin et al, 2010). However, at least one study has demonstrated that estrogens can decrease expression of CRH mRNA within the PVN (Grino et al; 1995) although Patchev and colleagues (1995) found that estrogen increases CRH mRNA in the PVN of ovariectomized (OVX) females, despite higher basal levels of PVN CRH mRNA in males. PVN CRH mRNA levels also appear to be higher in the afternoon of proestrus (Bohler et al, 1990) which would indicate some influence of circulating sex steroids on HPA axis activity.

The effects of androgens on HPA axis are perhaps more consistent than the reported effects of estrogen on HPA axis function, most likely due to the fact that this hormone shows relatively little, if any, fluctuation over time. Many rodent studies have supported the idea that androgens inhibit HPA axis activity (Williamson et al, 2005; 2010). For example, in a recent report, Williamson and colleagues (2010) provided evidence that in males, testosterone, specifically within the medial preoptic nucleus, can affect Fos expression in both the PVN and LS, as well as AVP mRNA expression in these regions. Furthermore, application of androgens in rhesus macaques reduces the CRH-stimulated CORT increase to the DEX/CRH test, consistent with the role of androgens inhibiting HPA axis function in non-human primates (Toufexis & Wilson, 2012).

**Effect of sex on stress responses in animals**

Rodent studies have also demonstrated that females have very different stress responses compared to males. For example, female and male rats exhibit different behavior (Albonetti & Farrabollini, 1995; Beck & Luine, 2002), blood pressure (Lopez-
Ruiz et al, 2008), conditioned fear (Mitsushima et al, 2006), stress-induced AVP (Jezova et al, 1996), central NE originating from the LC (Bangasser et al, 2011; 2012), peripheral catecholamines (Livezey et al, 1985; 1987; Weinstock et al, 1998; Uji et al, 2007), peripheral heat shock protein levels (Nickerson et al, 2006), and nociceptive responses (Aloisi et al, 1994; Gamaro et al, 1998) following acute stress. Male and female rats even exhibit differential long-term potentiation (LTP) recorded from hippocampal slices following acute stress (Huang et al, 2012). Furthermore, the DEX/CRF test appears to be an ethologically valid test for HPA axis function in rodent models, since rats bred selectively for high anxiety-like behavior appear to have both reduced DEX-induced suppression of ACTH and enhanced CRF-stimulated ACTH and CORT release, mimicking what is observed in depressed human patients (Keck et al, 2002). Finally, prior exposure to a severe, high intensity stressor can reduce the ability of DEX to suppress GCs following a subsequent mild stressor (novel environment) in male rats (O'Connor et al, 2003).

In addition, and of particular interest within this dissertation, it has been clear for many years that female rodents display markedly different HPA axis function than males, both at rest and following stress, although in rodents this effect appears to the opposite of what is observed in humans. Specifically, females have higher basal resting levels of ACTH and CORT (Kitay, 1961; Critchlow et al, 1963; Kitay 1963), and also have significantly higher stress-induced activation of the HPA axis following a wide variety of stressors. These stressors include footshock (Heinsbroek et al, 1991; Weinstock et al, 1998; Rivier, 1999), neonatal handling (Panagiotaropoulous et al, 2004), restraint (Le Mevel et al, 1978; 1979; Haleem et al, 1988; Aloisi et al, 1994; Aloisi
et al, 1998; Seale et al, 2004; Vieu et al, 2005), and immune challenge (Seale et al, 2004). Furthermore, maternal separation stress causes increased activation of CRF-producing neurons within the PVN of female, but not male, rats (Desbonnet et al, 2008). Taken together, it appears that the female rat HPA axis is more sensitive to stress-induced activation compared to the male rat HPA axis. Although this phenomenon in rodents is contradictory to stress-induced HPA axis sex differences seen in humans, there are a few possibilities for this discrepancy. First, most human studies measure salivary levels of cortisol, which may be misleading, since sampling salivary versus plasma levels of hormones can produce differential effects of sex in humans (Kudielka et al, 2004). In addition, as mentioned earlier, the TSST is essentially the only laboratory test used to investigate sex differences in stress responses, but this stressor may be fundamentally more stressful to men than to women, leading to higher observed stress responses in male subjects compared to female subjects.

The effect of sex and sex steroids on central stress neurocircuitry

Although CRF within the PVN is the central effector of stress-induced HPA axis activation, many brain regions are involved in the perception of stress, and especially of psychological stressors. Also, anatomical analysis of estrogen receptor (ER) distribution in the rat hypothalamus demonstrates that although the PVN does not contain ERα mRNA, ERβ is colocalized with CRF in the PVN, although this occurs only within the caudal portion of this region, and specifically not within CRF-containing neurons in the medial parvocellular PVN (Alves et al 1998; Laflamme et al, 1998). Furthermore, in a recent report, Williamson and colleagues (2010) provided evidence that in males, testosterone specifically applied to the medial preoptic nucleus can affect
activation of not only the PVN, but the lateral septum (LS) as well, in addition to arginine vasopression (AVP) mRNA expression in these regions and the medial nucleus of the amygdala (MeA). Taken together, this suggests that neither estradiol nor testosterone directly affect HPA axis hormone induction through PVN CRF-containing neurons following stress, and concurrent comparison of both androgen receptor and estrogen receptors within the male and female PVN has led others to the same conclusion (Bingham et al, 2006). Therefore the hypothesized mechanism for the control of HPA axis activity via gonadal hormones is that circulating estrogens and androgens can only indirectly affect HPA axis function, an idea that is supported by dense numbers of gonadal receptors found in extra-hypothalamic stress-responsive brain regions (Ostlund et al 2003; Bingham et al 2006; Byrnes et al 2009).

Sensitivity of the pituitary, adrenals, GC feedback mechanisms, contribution of other stress systems on HPA axis activity, and receptor distribution and/or function of glucocorticoid and/or sex steroids in many regions of the brain could all ultimately influence the magnitude of HPA axis output. Thus, there are many levels at which sex could influence HPA axis function, and research should be focused on the many extra-hypothalamic inputs that are sensitive to psychological stress. We have previously identified regions that are associated with HPA axis activation to audiogenic stress in male rats, including the ventrolateral septum (LSv), the anteroventral division of the bed nucleus of the stria terminalis (BSTav), the subiculum, and the medial preoptic area (MPOA); immediate early gene (IEG) expression in these regions was found to be highly correlated with PVN activity and HPA axis release of both ACTH and CORT (Burow et al, 2005). Others have implicated such regions as the medial prefrontal
cortex, the central nucleus of the amygdala (CeA) and medial nucleus of the amygdala (MeA) as limbic structures capable of affecting HPA axis responses to perceived threats (Emmert & Herman, 1999; 2003; Day et al, 2004). For example, Iwasaki-Sekino and colleagues (2009) observed higher basal and footshock stress-induced levels of CRH mRNA in the female PVN and CeA, although Viau and colleagues (2005) observed higher basal and restraint stress-induced CRF mRNA expression in the male CeA, and only a higher basal, but not stress-induced, level of PVN CRF expression in females following this stressor. Therefore, observed sex differences in stress-activated brain regions are not always consistent, as another study saw no significant effect of sex on IEG gene expression in the PVN, LS, or MeA 30 min after the termination of the same length of restraint stress (Figueiredo et al, 2002). Taken together, further analysis of central integrated stress-responsive neurocircuitry is warranted in order to understand the effects of sex and sex hormones on stress-induced HPA axis activity and on stress-related neurocircuitry in general in order to aid understanding of how sex may affect the perception of psychological stressors.

**Thesis Overview**

The experiments contained within this thesis aim to expand the knowledge of the effect of sex on physiological responses to psychological stressors, with a focus on central stress-responsive neurocircuitry. These studies utilize two different relatively mild psychological stressors, restraint and loud noise, to investigate the effect of sex on HPA axis hormone responses to both single and repeated presentations of these stressors. Chapter 2 focuses on the effects of sex on central stress neurocircuitry, specifically within known populations of CRF-producing neurons in response to acute
restraint stress, and to what extent endogenous circulating hormones in female rats are affecting these HPA axis hormone responses. It has been demonstrated that plasma levels of ACTH and CORT are useful in identifying the intensity of a psychological or emotional stressor (Kant et al, 1983; Armario et al, 1986; Burow et al, 2005), especially when these outcome measurements are used following mild to intermediate intensity stressors (Armario et al, 2012). Unlike restraint stress, noise is a stressor that allows for the variation of intensity by the use of different decibel levels of the noise. Chapter 3 investigates whether female rats have the same threshold of HPA axis activation with increasing, but still very mild, intensities of noise stress, and whether female rats display more prolonged HPA axis responses to a moderate intensity of noise stress compared to males. Furthermore, differential effects of sex on HPA axis hormones following acute presentations of two different stressor modalities, restraint and noise stress, are explored in the same animals in this chapter. Although many studies demonstrate clear effects of sex on responses to acute stress in rodents, one stressful experience is unlikely to cause psychiatric illness in humans (except if that experience is traumatic, as in the case of PTSD). Because most of the aforementioned studies utilized relatively mild psychological stressor, it may be more ethologically valid to measure the effect of sex on repeated exposures to stressors, since multiple mild stressors are more likely to occur to humans on a daily basis than the likelihood of experiencing a single stressor. Indeed, research has indicated that sex can also affect stress responses to repeated or chronic stress exposure. For example, Baran and colleagues (2009) observed differential effects of sex on the recall of fear conditioning and extinction following chronic restraint stress. Chapter 4 therefore explores the effect of sex on the extent to
which HPA axis hormones adapt to repeated presentations of either restraint or noise, which display stressor-specific effects on acute HPA axis activation. Chapter 5 provides an overall discussion of all the results herein, as well as indications for further research.
CHAPTER TWO:

SEX DIFFERENCES IN ACTIVATED CORTICOTROPIN-RELEASING FACTOR NEURONS WITHIN STRESS-RELATED NEUROCIRCUITRY AND HYPOTHALAMIC–PITUITARY–ADRENOCORTICAL AXIS HORMONES FOLLOWING RESTRAINT IN RATS

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Abstract

Women may be more vulnerable to certain stress-related psychiatric illnesses than men due to differences in hypothalamic-pituitary-adrenocortical (HPA) axis function. To investigate potential sex differences in forebrain regions associated with HPA axis activation in rats, these experiments utilized acute exposure to a psychological stressor. Male and female rats in various stages of the estrous cycle were exposed to 30 min of restraint, producing a robust HPA axis hormonal response in all animals, the magnitude of which was significantly higher in female rats. Although both male and female animals displayed equivalent c-fos expression in many brain regions known to be involved in the detection of threatening stimuli, three regions had significantly higher expression in females: the paraventricular nucleus of the hypothalamus (PVN), the anteroventral division of the bed nucleus of the stria terminalis (BSTav), and the medial preoptic area (MPOA). Dual fluorescence in-situ hybridization analysis of neurons containing c-fos and corticotropin-releasing factor (CRF) mRNA in these regions revealed significantly more c-fos and CRF single-labeled neurons, as well as significantly more double-labeled neurons in females. Surprisingly, there was no effect of the estrous cycle on any measure analyzed, and an additional experiment revealed no demonstrable effect of estradiol replacement following ovariectomy on HPA axis hormone induction following stress. Taken together, these data suggest sex differences in HPA axis activation in response to perceived threat may be influenced by specific populations of CRF neurons in key stress-related brain regions, the BSTav, MPOA, and PVN, which may be independent of circulating sex steroids.
Introduction

Stress can be an exacerbating or causal factor in the etiology of many diseases, including several psychological disorders. Some of these stress-influenced psychiatric illnesses are at least twice as prevalent in women than men, such as major depression (Linzer et al, 1996; Kessler et al, 2005; Van de Velde et al, 2010) and several anxiety disorders, such as posttraumatic stress and generalized anxiety disorders (Linzer et al, 1996; Stein et al, 2000; Holbrook et al, 2002; Tolin & Foa, 2006; Bekker & Van Mens-Verhulst, 2007; Olff et al, 2007; Christiansen & Elklit 2008; Vesga-Lopez et al, 2008; Ditlevesen & Elklit, 2010). In humans these disorders are associated with dysfunction, and specifically hyperactivity, of the hypothalamic-pituitary-adrenocortical (HPA) axis and thus the HPA axis is currently the target of therapeutic treatments for these illnesses (Lanfumey et al., 2008; Lloyd & Nemeroff, 2011). In the brain, the paraventricular nucleus of the hypothalamus (PVN) controls activation of the HPA axis in response to either real or perceived threats, and release of the hormones adrenocorticotropic hormone (ACTH) and cortisol. If hyperactivity of the HPA axis truly underlies stress-related psychiatric illness in humans, female susceptibility to these illnesses could potentially be explained by differences in HPA axis activation following perceived, or psychological, threats/stressor.

In rats, there is a wealth of evidence that females can have much larger magnitude HPA axis activation to stress than males. Female rats reportedly release more ACTH and corticosterone (CORT) compared to male rats following a wide variety of acute stressful stimuli (Le Mevel et al, 1978; 1979; Livezey et al, 1985; Heinsbroek et al, 1991; Aloisi et al, 1994; Handa et al, 1994; Ogilvie & Rivier, 1997; Weinstock et al,
In addition, activation of the PVN is significantly higher in females than males following various acute stressors, as indexed by either mRNA or protein products of the immediate early gene c-fos (Seale et al, 2004; Viau et al, 2005; Larkin et al, 2010). Presumably, sex biased stress-induced activation of the PVN, and subsequent HPA hormone induction are the result of corticotropin-releasing factor (CRF)-dependent differences, the primary PVN peptide controlling release of ACTH from the pituitary. Indeed, basal (Viau et al, 2005) and stress-induced CRF mRNA levels in the PVN have been reported to be higher in female compared to male rats (Aloisi et al, 1998; Iwasaki-Sekino et al, 2009). However, at least one group has reported the opposite effect after restraint stress (Sterrenburg et al, 2012), and Zavala and colleagues (2011) reported higher PVN c-fos (FOS) immunoreactivity in male compared to female rats after acute restraint. It remains unclear whether sex differences in PVN activation occurs specifically within CRF, or some other population, of neurons.

Very little research thus far has focused on sex differences in activation of brain regions associated with PVN relative activity following psychological stressors. However, uncovering how sex might influence these particular pathways may be especially important for understanding sex- and stress-influenced psychiatric disorders in humans. We have previously identified regions that are associated with HPA axis activation to psychological stress in male rats using audiogenic stress, including the ventrolateral septum (LSv), the anteroventral division of the bed nucleus of the stria terminalis (BSTav), the subiculum, and the medial preoptic area (MPOA), and c-fos mRNA expression in these regions was found to be highly correlated with PVN activity
and HPA axis hormone release (Burow et al, 2005). Others have implicated such regions as the medial prefrontal cortex and medial nucleus of the amygdala (MeA) as limbic structures capable of affecting HPA axis responses to perceived threats (Emmert & Herman 1999; Herman et al, 2003; Day et al, 2004). Importantly, several studies have shown sex differences in some of these regions. For example, Aloisi and colleagues (1997) observed sex differences in activation of the septal nuclei after either formalin injection or restraint stress. Females show less activity in the medial prefrontal cortex after inescapable tailshock than males, despite females having a greater HPA axis hormone induction following this stressor than males (Bland et al, 2005).

Of particular interest however, are potential sex differences in the BSTav and the MPOA that could affect HPA axis activity. Both of these regions have CRF-producing neurons, and they both contain a dense amount of both androgen and estrogen receptors (Simerly et al, 1990). Specifically, CRF-containing neurons in the fusiform nucleus of the BST send direct projections to the PVN (Dong et al, 2001). In addition, a sexually dimorphic population of CRF neurons exists in the MPOA (McDonald et al, 1994; Funabashi et al, 2004), which is a morphologically and functionally sexually differentiated region involved in the control of reproductive behavior containing dense amounts of steroid hormone receptors (Tobet & Hanna, 1997). Furthermore, this region has been found to be the site of inhibitory action of androgens on HPA axis activity in male rats (Viau & Meaney 1996; Williamson et al, 2010). Indeed, several researchers have named these particular regions as likely candidates for sex-specific influences on stress-induced HPA axis function (Viau 2002; Herman et al, 2005). However to date, no research has focused on stress-induced sex differences in these areas.
Therefore to investigate possible sex differences in stress-induced neurocircuitry following acute stress, we exposed male and female rats to 30 min of restraint. Because many studies have demonstrated estrous cycle influences on both basal (Atkinson & Waddell, 1997) and stress-induced (Viau & Meaney 1991; Rhodes et al, 2002; Conrad et al, 2004; Iwasaki-Sekino et al, 2009; Larkin et al, 2010) ACTH and CORT release, we included females in 3 stages of the estrous cycle: diestrus, proestrus, and estrus. In contrast to our previous studies in male rats using noise stress, we used restraint stress in this study due to the large volume of literature examining the effect of sex on responses to this stressor in particular. We first utilized the immediate early gene c-fos as a general marker of neuronal activation, in order to measure stress-induced brain activity across a wide selection of regions with dissimilar neuroanatomical characteristics. We then further investigated brain regions that were found to have a sex-specific activation to determine colocalization of CRF with c-fos using dual fluorescence in situ hybridization (FISH). Finally, we manipulated sex steroid levels in females and compared acute stress-induced HPA axis hormone activation in females with prolonged exposure to silastic capsules containing estradiol or vehicle, compared to intact male and female animals.

**Materials and Methods**

*Experiments 1 and 2: Animals*

Young adult (2-3 month old) Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) were allowed to acclimate in the colony for at least one week without manipulation upon arrival. All animals were originally group-housed but were
singly housed in the same room just prior to stress manipulation (Experiment 1), or following surgery (Experiment 2), and were maintained on a 12:12 h light:dark cycle (lights on at 6 am) under constant temperature and humidity conditions, and were provided access to food and water ad libitum. All experimental manipulations occurred between the hours of 8 am and 12 pm to control for diurnal rhythms of ACTH, CORT, and estradiol. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder and conformed to NIH guidelines.

**Experiment 1: Procedure**

All females were monitored daily for stage of estrous cycle via vaginal lavage. Only females exhibiting normal 4-5 day cycles were included in this study, and all females were monitored for at least 2 full cycles prior to stressor exposure. All males were handled daily in a similar fashion to control for this manipulation. To increase the likelihood of normal estrous cycling in the female subjects, cages were alternated by sex on racks in the colony room. Males and females predicted to be in each stage of the estrous cycle were run concurrently on each test day, over a total of 5 days. Prior to stressor exposure, animals were habituated to transport in their home cages from the colony to a behavioral testing suite down the hall from the colony room each morning for 5 days. Animals were left in an adjacent room to where restraint took place for 30 min. Similar to the previous 5 days, on the morning of the experiment, males (n = 6) and females that were predicted to be in the following estrous cycle stages: diestrus (n = 9; DI), proestrus (n = 8; PRO), and estrus (n = 6; EST) were transported to the behavioral suite and left untouched for at least 30 min. Animals were weighed and baseline blood samples were collected after which they were placed into Plexiglas tubes with tails
protruding, for a total of 30 min including basal blood sampling. Due to the significant body weight differences between males and females (data not shown; see Results 3.3), two different sized restrainers were used to achieve similar constraint of the animal within the tube. Males were restrained in tubes that were 20.6 cm in length and 6.3 cm in diameter, and females were restrained in tubes that were 18.1 cm in length and 5.1 cm diameter. The size of all tubes restricted gross movements in all directions, but did not interfere with animals’ breathing. All animals were sacrificed immediately following the 30 min of restraint.

**Experiment 2: Procedure**

Experiment 2 was carried out to further explore estrogenic influences on HPA axis responses to restraint stress in our lab. For this experiment, male and female Sprague-Dawley rats were raised in the animal colony at the University of Colorado at Boulder until they were 3 months of age. On days 1 and 2, females were ovariectomized via bilateral incisions and implanted with silastic capsules as described previously (Strom et al, 2008). Briefly, 30 mm long silastic tubing segments (Inner/Outer Diameter: 1.575/3.173 mm, Dow Corning, Fisher Scientific Inc, USA) were filled with 70 μl of either sesame oil vehicle (OVX; n = 10) or 17β-estradiol (E₂; 180μg/ml; Sigma) in sesame oil (OVX + E₂; n = 10), and were sealed with 5 mm long segments of 2 mm diameter wooden applicator sticks. Other female (intact females; n = 12) and male (intact males; n = 10) rats received sham surgery on the same days: identical bilateral incisions were made into the muscle walls and immediately sutured without removal of any tissues. Other than the day of surgery, all manipulations were performed between 8 am and 12 pm. Animals were given on week to recover from
surgery prior to further experimental manipulation. On days 9-14, following the surgical recovery period, rats were habituated to being transported down the hallway from the colony room to a behavioral testing suite. On day 10, prior to being transported to the behavioral suite (8-9 days post-surgery), basal blood samples were taken outside the colony room in which all animals were housed. On day 15 rats were exposed to a loud noise stressor for 30 min (data not shown). On day 17 (15-16 days post-surgery), all animals were again transported from the colony room to the behavioral testing suite and left untouched for at least 15 min, but were then exposed to 30 min of restraint just as in Experiment 1, immediately after which all animals were sacrificed. On the day of restraint males weighed 367 ± 5.34 g and females weighed 236 ± 3.05 g, so two different sized restrainers were used as in Experiment 1.

Experiments 1 and 2: Blood and Tissue Collection

Blood samples before sacrifice were collected from a lateral tail vein. Briefly, animals were removed from their home cages and gently restrained in a towel. A puncture was then made with the corner of a razor blade into one of the lateral tail veins. Samples for ACTH analysis (300-400 µl) were collected into plain glass capillary tubes and placed into chilled tubes containing 15 µl of ethylenediaminetetraacetic acid (EDTA; 20 mg/ml). Samples for estradiol and CORT analyses (100-200 µl for experiment 1; 300-400 µl for Experiment 2) were collected into heparinized glass capillary tubes and placed into empty chilled microcentrifuge tubes. The entire procedure lasted less than 5 min per animal to avoid detecting rising hormone levels due to the procedure itself. Whole blood samples were centrifuged for 2 min at 14,000 rpm and plasma for each hormone was extracted and stored at -80°C. Following
decapitation, blood samples and brain tissue were harvested simultaneously. Trunk blood was collected into chilled EDTA-coated plastic tubes (Vacutainer® EDTA(K2) tubes; BD Diagnostics, Franklin Lakes, NJ) for determination of ACTH, CORT, and estradiol concentration. Whole blood Vacutainers were then centrifuged at 4°C for 10 min at 2,000 rpm, after which plasma was extracted and stored at -80°C. Brains were rapidly removed, frozen in chilled methylbutane (between -20 and -30°C), and stored at -80°C until further processing.

Experiments 1 and 2: ACTH Immunoradiometric Assay (IRMA)

Plasma ACTH concentrations were assayed using a commercially available immunoradiometric assay kit (Cat # 27130; DiaSorin, Stillwater, MN, USA); which has a sensitivity of 1.5 pg/ml, and inter- and intra-assay coefficients of ~4.6% and ~ 4.18%, respectively. This IRMA kit recognizes 100% of whole molecule ACTH (detects all 39 amino acids), and as such is very specific, with undetectable cross-reactivity for α- and β-MSH, as well as β-Endorphin. All samples from each experiment were analyzed separately within one assay to eliminate interassay variability. The assay was performed according to the kit directions. Briefly, 200 µl of plasma or plasma diluted with zero standard (provided with the kit) was incubated overnight with a 125I-labelled monoclonal antibody specific for ACTH amino acids 1-17, a goat polyclonal antibody specific for ACTH amino acids 26-39, and a polystyrene bead coated with a mouse anti-goat IgG. Only ACTH in the sample with all 39 amino acids bound both antibodies to form an antibody complex on the polystyrene bead. The following day, tubes containing the radioactive beads were washed to remove any unbound reagents and the amount of radioactivity still left on each bead was determined with a gamma counter. The
concentration of ACTH for each sample was calculated by fitting values for each sample into a standard curve of known ACTH dilutions processed concurrently.

*Experiments 1 and 2: CORT Enzyme-linked Immunosorbent Assay (ELISA)*

Plasma CORT concentrations were analyzed using a commercially available kit (Cat. # 901-097; Assay Designs, Ann Arbor, MI, USA). This is a highly specific assay (100% cross-reactivity with CORT, 28.6% with deoxycorticosterone, and 1.7% with progesterone), with a sensitivity of 26.99 pg/ml, and inter-/intra-assay variability of ~7.5% and ~9.7%, respectively. All samples from each experiment were analyzed within one assay to eliminate interassay variability. The kit directions were followed with the exception of a modification to use a smaller volume of plasma as follows. The steroid displacement reagent (0.5 µl/ml; provided with the kit) was added to the assay buffer. Ten µl of plasma was then diluted 1:50 with the amended assay buffer. The diluted plasma samples were then processed according to the kit directions. This method was found to result in assayed CORT levels equivalent to the method in the kit directions (data not shown). Briefly, plasma samples and diluted CORT standards were incubated in a 96-well plate coated with anti-sheep antibody raised in donkey, together with CORT with a covalently attached alkaline phosphatase molecule, and a sheep polyclonal antibody for CORT. After a 2 hr incubation period the plate was washed to remove all unbound reagents and a substrate was added. After 1 hr the color reaction was stopped and the intensity of color in each well was analyzed at 405 nm using a microplate reader (Biotek EL808; Winooski, VT, USA). The concentration of CORT for each sample was calculated by fitting unknown values into a standard curve of known CORT dilutions processed concurrently.
Experiments 1 and 2: Estradiol Radioimmunoassay (RIA)

Plasma estradiol concentrations were determined using a double-antibody radioimmunoassay kit (Cat # KE2D1; Siemens Healthcare Diagnostics, Los Angeles, CA). The antiserum used in this kit is highly specific for estradiol (100% detection of 17β-estradiol) and has virtually undetectable cross-reactivity with other naturally occurring steroids; the highest occurring cross-reactivity being with estrone at 12.5%. The sensitivity of this assay is 1.4 pg/mL, has intra-assay variability of 4-14% and inter-assay variability of 3.5-5.5%. However, all samples were run in a single assay to eliminate interassay variability. The assay was performed according to kit directions. Briefly, samples, calibrators, and controls were first incubated with anti-estradiol serum for 2 hr, after which \(^{125}\text{I}\)-labeled estradiol is added which competes for antibody sites with estradiol already in the tubes for 1 hr. Separation of the bound estradiol from the free estradiol was then achieved by a PEG-accelerated double-antibody method. Then, the antibody-bound fraction was precipitated by centrifugation at 4°C for 20 min, the supernatant was aspirated, and the remaining pellet was counted on a gamma counter. Unknown concentrations of estradiol in the samples were then calculated using a calibration curve run concurrently.

Experiment 1: Radioactive In Situ Hybridization

The method for in situ hybridization has been described previously (Day et al, 2005). Briefly, whole brains stored at -80°C were mounted on chucks at -20°C. Ten micrometer thick sections were cut on a cryostat (Model 1850; Leica Microsystems, Buffalo Grove, IL, USA), thaw-mounted onto polylysine-coated slides and stored at -
80°C. Slides were taken from storage and immediately fixed in cold 4% paraformaldehyde for 1 hr, acetylated in 0.1M triethanolamine (pH 8.0) with 0.25% acetic anhydride for 10 min, dehydrated through graded ethanols to 100% and air dried. A riboprobe against c-fos mRNA (680 mer; courtesy of Dr. T. Curran, St. Jude Children’s Hospital, Memphis, TN) was generated using standard transcriptional methods and labeled with $^{35}$S-UTP (Perkin Elmer, Waltham, MA). Brain sections were hybridized overnight at 55°C with the riboprobe diluted to 1-3 $\times 10^6$ c.p.m. per 70 µl in hybridization buffer containing 50% formamide, 10% dextran sulfate, 3X saline sodium citrate, 50mM sodium phosphate (pH 7.4), 1X Denhardt’s solution, 0.1 mg/ml yeast tRNA, and 10mM dithiothreitol. The following day, the probe was washed from the slides, and they were then treated with RNase A (200 µg/ml; pH 8.0) at 37°C for 1 hr, washed to a final stringency of 0.1X saline sodium citrate for 1 hr at 65°C, dehydrated again in graded ethanols to 100%, and air dried. Slides were then exposed to X-ray film (BioMax-MR; Eastman Kodak, Rochester, NY) for 7-12 days. Films were then analyzed as described below.

Levels of c-fos mRNA were analyzed by computer-assisted optical densitometry by an experimenter blind to the treatment conditions. Images of each individual brain section were captured digitally (CCD camera, model XC-77; Sony, Toyko, Japan), and digital images were then analyzed using Scion Image (Version 4.03 for Windows; ScionCorp). First, the relative optical density of the x-ray film was determined using a macro within Scion Image (written by Dr. S. Campeau) which allowed the automatic determination of a signal above background. Specifically, for each section, a background sample was taken over an area of white matter, and the signal threshold
was set as 3.5 standard deviations above the mean gray value of the background. The remaining pixels above this threshold were then analyzed within the brain region of interest. It should be noted that although background criteria were relatively stringent, this method still results in a few pixels above background in areas on the x-ray film not on the brain section, indicating that even weak intensity mRNA signal on the tissue is detected. For consistency, a different template was created for each brain region, and was placed using anatomical landmarks based on the white matter distribution of the unstained tissue, according to a standard rat brain atlas (Paxinos & Watson, 2005). The number of pixels above background was multiplied by the signal above background to give an integrated density value for both hemispheres throughout the rostral-caudal extent of each brain region of interest. This method has been reported to reflect both the number of cells expressing mRNA and the expression level per cell, as determined by cell and grain counts of emulsion-dipped slide (Day et al, 2005). The mean integrated density for each animal was then calculated by averaging the highest 2 or 3 values for each hemisphere depending on the brain region resulting in a single value for each animal representing the peak of c-fos mRNA expression for each brain region of interest.

**Experiment 1: Dual Fluorescence In Situ Hybridization (FISH)**

In a subset of animals, adjacent sections to those used for radioactive in situ hybridization determination of c-fos mRNA expression were also analyzed using fluorescent in situ hybridization (FISH). This procedure allowed for colocalization of c-fos and CRF mRNA expression within the same cells within brain regions where a sex difference was seen using traditional radioactive in situ hybridization analysis of c-fos.
mRNA expression. Initial slide processing and probe hybridization was performed using the same procedure described above for radioactive in situ hybridization, except for the following differences. Riboprobes against c-fos mRNA (described above) or CRF mRNA (770 mer; cDNA provided by Dr. R. Thompson at the University of Michigan) were both synthesized using T7 RNA polymerase with fluorescein-12-UTP and digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN), respectively. A total of 6-8µl of each labeled probe was added to each slide in hybridization buffer (details above) containing 20mM DTT. After the high stringency wash, slides were placed into 0.05M phosphate-buffered saline (pH 7.4; PBS) overnight at 4°C. On the following day, endogenous peroxidase was quenched in 2% H₂O₂ in PBS for 30 min at room temperature with gentle agitation, after which slides were washed with 1X Tris-buffered saline containing 0.05% Tween-20 (pH 7.5; TBS-T). After a 30 min incubation at room temperature in blocking buffer (FP1012; Perkin Elmer, Waltham, MA), slides were incubated with anti-fluorescein-HRP (1:250 in blocking buffer, 80µl/slide; NEF710, Perkin Elmer, Waltham, MA) in humidified chambers for 30 min. After removing coverslips and washing slides in TBS-T, the fluorescein-UTP-Fos complex was detected using a tyramide signal amplification kit with fluorescein as the fluorophore for 1 hr at room temperature in a humidified chamber (1:100, 80µl/slide; TSA-Plus Kit, Perkin Elmer, Waltham, MA). Slides were washed in TBS-T, rinsed in 1X TBS (pH 7.5; no Tween) to remove residual detergent, and transferred to PBS. After repeating the endogenous peroxidase quenching procedure, slides were washed with TBS-T, and then incubated with anti-digoxigenin-peroxidase (1:500 in blocking buffer, 80µl/slide; Roche Applied Science, Indianapolis, IN) for 30 min at room temperature in humidified
chambers. After rinsing with TBS-T, the digoxigenin-UTP-CRF complex was then visualized using Cyanine-3 as the fluorophore as above. After the final tyramide amplification similar to that described above, slides were rinsed with TBS-T, then TBS, and then coverslipped with Vectashield hardset mounting medium (containing DAPI as a counterstain; H-1500, Vector Laboratories, Burlingame, CA). Control slides of the same tissue run without addition of probe or without amplification resulted in sections with no detectable fluorescence.

Cells containing fluorescent markers for c-fos and CRF mRNA were visualized with an upright fluorescence microscope (AxioImager Z1; Zeiss Microscopy, Thornwood, NY, USA). Single- and double-labeled cells were counted using AxioVision software (v. 4.8.2) tools ‘aligned rectangle’ placed according to landmarks seen using DAPI counterstaining in order to analyze a consistently sized area between animals, and the ‘measure events’ tool to prevent multiple counts of the same cell.

**Experiment 1: Statistical Analysis**

Body weight data for Experiment 1 were analyzed with a one-way analysis of variance (ANOVA) with males and females in each stage of the estrous cycle. Baseline and stress-induced ACTH and CORT concentrations were analyzed by repeated measures ANOVA, and independent samples t-tests were run on hormone data before and after stress to determine the source of significant interactions. Endogenous estradiol concentrations in female rats were analyzed using a one-way ANOVA, and an independent samples t-test was used to test for a sex difference. To test the possible effect of the estrous cycle on c-fos mRNA expression across all brain regions, data in
female rats were analyzed using a one-way ANOVA. To test whether a significant sex difference in c-fos mRNA expression levels across brain regions existed, log-transformed data were analyzed using independent samples t-tests. Post-hoc analyses, when necessary, were performed using Tukey’s HSD multiple means comparisons. Significance was set at $p < 0.05$ for analysis of body weight and hormone data. To further reduce the possibility of Type I error when analyzing c-fos mRNA expression data from several brain regions, statistical significance for these tests was set at $p < 0.01$. FISH-derived cell counts were first analyzed with one-way ANOVAs in females only to test for estrous cycle effects. Sex differences in these data were then analyzed with subsequent t-tests, with significance set at a $p$ value of 0.05.

**Experiment 2: Statistical Analysis**

ACTH and CORT data were analyzed with repeated measures ANOVA. Significant main effects in the repeated measures ANOVAs were explored further with one-way ANOVAs. Estradiol concentrations 8-9 days post-surgery and 15-16 days post-surgery were analyzed via one-way ANOVAs separately. Further post-hoc analyses, if necessary, were performed using Tukey’s HSD multiple means comparisons. Significance was set at $p < 0.05$ for all statistical tests for experiment 2. PASW Statistics (formerly SPSS, version 18 for Windows) was used for all statistical analyses.

**Results**

**Experiment 1: HPA Axis Hormones**
Figure 2.1 displays HPA axis hormone plasma concentrations immediately prior to and immediately following, 30 min of restraint. Repeated measures ANOVA revealed that restraint significantly increased ACTH levels in both males and females as

**Figure 2.1**

HPA axis hormone concentrations immediately prior to (Before Stress) and following (After Stress) 30 min of restraint stress in Experiment 1. All values represent group means ± 1 SEM. (A,B) Plasma concentrations of ACTH (A) and CORT (B) in intact male and female rats. * P < 0.05 compared to males at the same timepoint. (C,D) Plasma concentrations of ACTH (C) and CORT (D) in females across three stages of the estrous cycle. *P < 0.05 compared to stress condition within the same estrous cycle stage.
reflected by a main effect of stress ($F_{1,27} = 46.54$, $p < 0.001$; Fig 2.1A). In addition, females had higher ACTH levels compared to males, as revealed by a significant main effect of sex ($F_{1,27} = 13.59$, $p = 0.001$). There was also a significant stress by sex interaction ($F_{1,27} = 7.39$, $p = 0.01$). Post-hoc analyses revealed that females had significantly higher concentrations of ACTH only after stress ($t(27) = 3.32$, $p < 0.01$) and did not differ significantly before stress ($t(27) = 1.33$, $p = 0.19$). Restraint also significantly increased CORT concentration in both males and females, as revealed by a significant main effect of stress ($F_{1,27} = 81.43$, $p < 0.001$; Figure 2.1B). Overall, females also had significantly higher concentrations of CORT compared to males ($F_{1,27} = 17.12$, $p < 0.001$). In addition, there was also a significant stress by sex interaction on CORT concentration ($F_{1,27} = 6.04$, $p = 0.02$). Post-hoc analyses revealed that although females had significantly higher CORT concentrations before and after stress, the effect of sex on CORT concentration was larger after restraint ($t(27) = 3.73$, $p = 0.001$) than before restraint ($t(27) = 2.2$, $p = 0.03$).

Restraint significantly increased ACTH concentration ($F_{1,20} = 101.62$, $p < 0.001$; Fig 2.1C) and CORT concentration ($F_{1,20} = 153.49$, $p < 0.001$; Fig 2.1D) in all females. However, there was no main effect of estrous cycle stage on either ACTH ($F_{2,20} = 0.63$, $p = 0.54$) or CORT ($F_{2,20} = 1.06$, $p = 0.37$) concentrations. In addition there was not a significant stress by estrous cycle interaction on either ACTH ($p = 0.28$) or CORT ($p = 0.35$) concentration.

**Experiment 1: Plasma Estradiol Concentration**

Figure 2.2A displays plasma estradiol concentrations for all groups from Experiment 1. There was a significant effect of group ($F_{3,25} = 7.99$, $p = 0.001$) on
estradiol concentration. Post-hoc analyses revealed that females in proestrus and in estrus had significantly higher estradiol concentrations than male rats ($p$'s < 0.05), and females in proestrus also had significantly higher estradiol concentration than females in diestrus ($p = 0.005$). Females in proestrus did not differ significantly from females in estrus ($p = 0.68$), and females in diestrus did not differ significantly from males ($p = 0.82$).

**Experiment 1: In situ Hybridization c-fos mRNA Expression**

As expected, female rats displayed c-fos mRNA expression in all of the regions analyzed, indicating a qualitatively similar pattern of brain activation in male and female rats overall in response to restraint stress. Within females only, there was no effect of the estrous cycle on c-fos mRNA expression in any brain region analyzed, and so data were pooled for females across estrous cycle. Body weight analysis revealed a main effect ($F_{3,25} = 129.73$, $p < 0.001$; data not shown), and post-hoc tests verified that males weighed significantly more than females in each stage of the estrous cycle ($p$'s < 0.001). There was no significant difference in body weight across estrous cycle ($p$'s > 0.05; data not shown). Due to the significant difference in body weights, and to determine if this affected somatosensory experience of stress, the following brain regions were analyzed because they reflect the somatosensory processing of restraint stress: the cuneate nucleus within the brainstem (CUN), the ventroposterolateral and ventroposteromedial nuclei of the thalamus (VPL/VPM), and the barrel field region of the primary somatosensory cortex (S1BF). There was no significant difference between males and females for any of the sensory areas analyzed ($p$'s > 0.01; data not shown). The remaining regions were analyzed due to their high correlations with stress-induced
ACTH and c-fos mRNA expression in the PVN as demonstrated previously in male rats following loud noise stress (Burow et al, 2005), or due to their reported involvement in stress responses. There was no significant effect of sex on stress-induced c-fos mRNA induction in the following regions (p’s > 0.01; data not shown): the anterior medial prefrontal cortex (AntmPFC), the prelimbic region of the prefrontal cortex (PL), the infralimbic region of the prefrontal cortex (IL), the oval nucleus of the bed nucleus of the stria terminalis (BSTov), the lateral septum (LS), the septohypothalamic area (SHy), the dorsomedial nucleus of the hypothalamus (DMH), the medial nucleus of the amygdala (MeA), and the basolateral nucleus of the amygdala (BLA). However, female rats did display significantly higher c-fos mRNA expression compared to male rats in the following brain regions: the BSTav (t(27) = 4.2, p < 0.001; Figure 2.2 B), the MPOA (t(27) = 3.3, p = 0.002; Figure 2.2 C), and the PVN (t(27) = 3.8, p = 0.001; Figure 2.2 D). Note that the pattern of c-fos mRNA expression seen across the estrous cycle in these 3 regions (Figure 2.2 B-D) does not mimic plasma estradiol levels (Figure 2.2 A). Figure 2.3 depicts representative photomicrographs of brain regions from both sexes that exhibited higher c-fos mRNA expression in females compared to males.
Figure 2.2

All values represent group mean ± 1 SEM. A: Plasma estradiol concentrations on the day of stress in Experiment 1. *P < 0.05 compared to males. #P < 0.05 compared to DI. B, C, D: Relative c-fos mRNA expression immediately after stress in Experiment 1 within the BSTav (B), the MPOA (C), and the PVN (D). *p < 0.05 for all females compared to males. Abbreviations: BSTav – Bed Nucleus of the Stria Terminalis; MPOA – Medial Preoptic Area; PVN – Paraventricular Nucleus of the Hypothalamus; DI – diestrus; PRO – proestrus; EST – estrus.
Figure 2.3

Representative photomicrographs displaying the effect of sex on c-fos mRNA expression of female (A,B,C) and male (D,E,F) brains; within the anteroventral region of the bed nucleus of the stria terminalis (BSTav; A,D), the medial preoptic area (MPOA; B,E), and the paraventricular nucleus of the hypothalamus (PVN; C,F).

Experiment 1: Dual FISH CRF and c-fos mRNA expression

Adjacent sections from within the regions where a significant effect of sex was seen with in situ quantification of FOS alone (BSTav, MPOA, and PVN) within a subset of rats from Experiment 1 were also analyzed for double-labeling of CRF and c-fos (FOS) mRNAs. Figure 2.4 displays examples of FISH labeled brain sections from areas analyzed, with red boxes in A-C approximating the Bregma locations of photomicrographs in D-I (Paxinos and Watson, 2005). Figure 2.4 panels D, E, and F
are photomicrographs taken from a representative female brain, and panels G, H, and I exhibit the same for a representative male brain. In this figure, blue color represents a control stain for DNA material in order to show cell nuclei, the color red represents CRF single-labeling, a green color represents single-labeling for FOS, and the color yellow indicates areas of overlapping green and red, which represents cells double-labeled for CRF and FOS. Qualitatively, the BSTav and MPOA contained not only drastically fewer labeled cells as the PVN, but the cells in these regions were also much more sparse compared to the densely packed cells of the PVN, although all 3 regions exhibited cells labeled green, red, and yellow.

No effect of the estrous cycle was observed on any FISH measure quantified (p’s > 0.05; data not shown), therefore all subsequent analyses for sex differences compared all females collapsed across estrous cycle to males. As demonstrated in Figure 2.5, females had significantly more FOS-labeled cells in the BSTav (t(18) = 3.8, p < 0.01), MPOA (t(18) = 6.0, p < 0.0001), and PVN (t(18) = 4.3, p < 0.001). In all three of these regions, the number of cells labeled for FOS was approximately 2-fold higher in the female compared to male brain, which was qualitatively similar to the increased expression observed via in situ hybridization (see Figure 2.2). Significantly more CRF-labeled neurons were also observed in the female BSTav (t(18) = 3.2, p < 0.01), MPOA (t(18) = 2.7, p < 0.05), and PVN (t(18) = 3.0, p < 0.01) compared to the male brain, although the magnitude of this difference was not as large as the number of FOS-labeled neurons. Importantly, the number of CRF and FOS double-labeled neurons was also significantly higher in the female BSTav (t(18) = 2.4, p < 0.05), MPOA (t(18) = 4.3, p < 0.001), and PVN (t(18) = 5.1, p < 0.0001) compared to the male brain. Finally,
Figure 2.4

Representative photomicrographs of c-fos and CRF mRNA labeled neurons using dual FISH in the BSTav (A,D,G), MPOA (B,E,H) and PVN (C,F,I). A,B,C: Representative Bregma images with red boxes showing the approximate location of photomicrographs in D-I. D,E,F: Magnified images from a female brain; Scale bar in D = 50µm. G,H,I: Images of a representative male brain at same magnification as in D,E,F. Blue=DAPI stain for nuclei; Red=CRF mRNA; Green=c-fos mRNA; Yellow= overlap of CRF and c-fos mRNA.

the female BSTav ($t(18) = 3.5$, $p < 0.01$) and MPOA ($t(18) = 5.3$, $p < 0.001$) had significantly more FOS-labeled neurons that did not contain CRF compared to the male brain, but there was no significant sex difference in this cell type in the PVN ($p > 0.05$). Indeed, only within the PVN did females exhibit a higher percentage ($t(18) = 3.6$, $p < 0.01$) of FOS neurons containing CRF than males (data not shown).
Figure 2.5

The effect of sex on CRF and c-fos mRNA containing cells as visualized by FISH in Experiment 1, and colocalization of both genes in the BSTav (A), the MPOA (B), and the PVN (C) after 30 min of restraint. * = p < 0.05 compared to males.
Experiment 2

All data from Experiment 2 are presented in Figure 2.6. Repeated measures ANOVAs on plasma hormone concentrations revealed that restraint stress significantly increased both ACTH \((F_{1,38} = 78.88, p < 0.001; \text{Figure 2.6 A})\) and CORT concentrations \((F_{1,38} = 190.69, p < 0.001; \text{Figure 2.6 B})\). A significant interaction between stress condition and treatment group existed for ACTH concentrations \((F_{3,38} = 5.88, p = 0.002)\), and post-hoc tests revealed a significant effect of group only after stress \((F_{3,38} = 6.4, p = 0.001)\). Specifically, OVX females had significantly higher ACTH concentration than all other groups following restraint stress \((p's < 0.05)\), but none of the other groups differed significantly from each other \((\text{Figure 2.6 A})\). There was a significant main effect of treatment group on basal \((F_{3,38} = 3.34, p = 0.03)\), but not stress-induced CORT concentrations \((\text{Figure 2.6 B})\). Specifically, intact females and OVX + E\(_2\) females had significantly higher basal CORT concentrations than males, and OVX + E\(_2\) females were also significantly higher than OVX females \((p's < 0.05)\). Male and OVX females’, as well as intact and OVX females’ CORT concentrations did not differ significantly \((p's > 0.05)\).

Ovariectomy alone or with estradiol replacement had a robust effect on estradiol concentrations \((\text{Figure 2.6C})\). Estradiol concentrations in male rats were only analyzed following restraint stress, due to the assumed stability of the concentration of this hormone in these animals. One week after ovariectomy (Basal), there was a significant effect of treatment group on estradiol concentrations \((F_{2,29} = 59.90, p < 0.001)\). Ovariectomy significantly decreased estradiol concentration compared to intact females \((p = 0.02)\), whereas ovariectomy with estradiol replacement \((\text{OVX} + \text{E}_2)\) significantly increased estradiol concentrations compared to OVX and intact females \((p's < 0.001)\).
Following restraint stress (15-16 days post-surgery; Stress Group), there was still a significant effect of treatment group on estradiol concentrations ($F_{3,38} = 113.19, p < 0.001$). OVX + E$_2$ females had significantly higher estradiol concentrations than all other groups, and intact females also had significantly higher estradiol concentrations than males and OVX females ($p$'s < 0.001). Males and OVX females did not have significantly different estradiol concentrations ($p > 0.05$). In the intact females, there was no significant effect of stage of estrous cycle on plasma ACTH, CORT or E$_2$ ($p$'s > 0.05; data not shown).
Figure 2.6

Experiment 2: Plasma hormone concentrations (means ± 1SEM) from intact males (n = 10) and females (n = 12) given sham surgery, and ovariectomized females with (OVX + E2; n = 10) or without (OVX; n = 10) estradiol replacement at baseline (Basal) and after 30 min of restraint (Stress). * = p < 0.05 compared to all other groups within the same stress condition. # = p < 0.05 compared to intact males and OVX females within that stress condition.
Discussion

The fact that HPA axis activation in response to a variety of stressful stimuli can be affected by sex and sex steroids is well established; the exact mechanism by, and level at, which this can occur remains obscure. The present study focuses on the forebrain neural circuit associated with stress-induced HPA axis activation, whether sex differences in stress-induced HPA axis hormone induction are accompanied by widespread increases in activation across neural circuits known to be involved in processing of psychological stressors, and the extent to which HPA axis hormone induction in females can be attributed to activational, or concurrent, effects of endogenous or exogenous sex steroids. To our knowledge, no previous studies have investigated sex differences in extended neural circuits, and specifically extra-PVN CRF populations known to influence subsequent HPA axis responses following acute psychological stress. Similar to previous findings using restraint as a psychological stressor (Seale et al 2004; Iwasaki-Sekino 2009; Larkin et al, 2010), females in the present study had significantly higher concentrations of ACTH and CORT following 30 min of restraint in addition to significantly higher activation of the PVN compared to male rats (Figure 2.1 A&B; Figure 2.2 D, respectively). In addition to central HPA axis differences, many regions we have previously linked to HPA axis activation following psychological stress in male rats (Burow et al 2005) were also activated in the female brain, including the LS, septohypothalamic area, infra- and pre-limbic regions of the prefrontal cortex, dorsomedial nucleus of the hypothalamus, and several stress-responsive amygdalar nuclei (data not shown).
The generally accepted, current hypothesized mechanism of sex differences in stress-induced HPA axis function is that estrogens stimulate, and androgens inhibit, this neuroendocrine axis by directly affecting its' central effector, the PVN. PVN CRF mRNA levels appear to be higher in the afternoon of proestrus (Bohler et al, 1990), and Patchev and colleagues (1995) found that estrogen increases CRF mRNA in the PVN of OVX females, despite higher basal levels of PVN CRF mRNA in males, which would indicate some influence of circulating sex steroids on HPA axis activity. In rhesus monkeys, simulation of the follicular phase of the menstrual cycle increased CRF mRNA in the PVN of OVX females, but simulation of the luteal phase produced no change from controls (Roy et al, 1999). Indeed, the human CRF gene contains estrogen response elements in the promoter region, supporting the idea that circulating sex steroids could directly regulate CRF expression and HPA axis activity (Vamvakopoulos & Chrousos 1993). Our data suggest that despite clear sex differences, stage of estrous cycle does not invariably have a large influence on HPA axis hormone responses or brain activation following acute restraint stress (Figure 2.1 C&D; Figure 2.2, respectively). A few other reports of null effects of estrous cycle on stress responses also exist (Guo et al, 1994; Rivier 1999; Bland et al, 2005). In Experiment 1, there was no effect of the estrous cycle on basal or stress-induced ACTH or CORT concentrations, or on c-fos mRNA expression in the PVN. If estradiol had a truly augmenting, activational effect on stress-induced HPA axis activation, females in PRO, with the highest estradiol concentrations (Figure 2.2 A), would be expected to have the highest ACTH, CORT, and PVN c-fos mRNA expression, as well as more c-fos and CRF double-labeled neurons in the PVN, but they did not differ significantly from females in other estrous
cycle stages on any of these measures. Estrous cycle also did not affect any measures in the PVN afferents analyzed, the BSTav and MPOA. Furthermore, estradiol concentrations in all animals (males included) were not significantly correlated with ACTH or CORT concentrations, or c-fos mRNA expression in any brain region (data not shown). Furthermore, anatomical analysis of estrogen receptor (ER) distribution in the rat hypothalamus demonstrates that although the PVN does not contain ERα mRNA, ERβ is highly colocalized with CRF, although this occurs only within the caudal portion of this region, and specifically not within CRF-containing neurons in the medial parvocellular PVN (Alves et al, 1998; Laflamme et al, 1998), suggesting that estradiol cannot directly affect HPA axis hormone induction through PVN CRF-containing neurons following stress, and concurrent comparison of both androgen receptor and estrogen receptor within the male and female PVN has led others to the same conclusion (Bingham et al, 2006).

Our data do support the idea though, that sex may affect development (i.e. the organization) of stress neurocircuitry, since overall female compared to male rats exhibited higher c-fos mRNA expression in several brain regions, despite no evidence of activational effects of circulating sex steroids. Not only did females overall exhibit significantly more single-labeled FOS and CRF neurons within the PVN, but also significantly more neurons double-labeled with both FOS and CRF. Furthermore, the augmented number of activated neurons in the female PVN can be attributed to increased activation of specifically CRF-producing neuroendocrine cells, as there was no significant sex difference in the number of non-CRF-containing cells double-labeled with FOS, and the percentage of FOS-labeled neurons also labeled with CRF was
significantly higher in the female PVN (Figure 2.5 C). Zavala and colleagues (2011) found no effect of sex in cells containing both FOS and AVP, but did observe that females had significantly higher FOS in cells that also contained glucocorticoid receptors, which mimics the pattern of FOS and CRF co-expression observed here. Because almost all CRF-expressing neurons in the medial parvocellular PVN also express glucocorticoid receptors (Liposits et al, 1987; Ceccatelli et al, 1989), the results of Zavala et al (2011) with those presented here are likely synonymous.

It is also possible sex steroids indirectly affect HPA axis function due to dense numbers of estrogen and androgen receptors in stress-responsive brain regions other than the PVN (Ostlund et al, 2003; Bingham et al, 2006; Byrnes et al, 2009), given the increased activation of the BSTav and MPOA observed in the female brain, regions which both send direct projections to the PVN (Cullinan et al, 1993; Campeau & Watson 2000; Forray & Gysling 2004; Radley et al, 2009; Radley & Sawchenko 2011). We previously found that c-fos mRNA expression in the anteroventral region of both the BST and the MPOA correlate highly with ACTH and CORT concentrations as well as with c-fos mRNA expression in the PVN following acute audiogenic stress in male rats (Burow et al, 2005). In addition, ERα and ERβ are abundant in both of these regions (Laflamme et al, 1998), implying that these regions are poised at an ideal position for estrogens to indirectly influence the PVN. Alternatively, a recent report by Williamson and colleagues (2010) provided evidence that in males, testosterone, specifically within the MPOA, can affect Fos expression in both the PVN and the LS, as well as arginine vasopression (AVP) mRNA expression in these regions and the MeA. And since androgen receptor-containing neurons projecting directly to the PVN are found in both
the male BSTav and MPOA (Williamson and Viau, 2007), it is likely that these regions could also be important for female sex steroids to influence HPA axis activity as well. Lesion studies in male rats support the idea that anteroventral nuclei of the BST are involved in excitation of the HPA axis (Choi et al, 2007; 2008), and since we observed significantly higher activation of this region in females, this could be a direct mechanism also contributing to augmented HPA axis hormone induction following stress in females. Chemoarchitectural analysis of the male BST illustrates the fusiform nucleus as having the densest population of CRF neurons within the anteroventral region, in stark contrast to the surrounding nuclei (Ju et al, 1989), and we demonstrate that females have significantly more CRF neurons in this area. CRF neurons in the BSTav receive direct innervations from both serotonin (Phelix et al, 1992) and norepinephrine (Phelix et al, 1994) cell groups. Here we also confirm previous reports of a larger population of CRF neurons within the female MPOA (McDonald et al, 1994; Funabashi et al, 2004). Though, because the majority of activated cells in both the female BSTav and MPOA occurred mostly in cells that do not contain CRF, we cannot conclude that direct activation of CRF-containing neurons in these areas are essential for higher HPA axis output observed in females. Demonstrated here is that these regions are indeed more highly activated in the female brain in response to restraint, indicating that these regions may indeed underlie sex differences in HPA axis function, perhaps through some other cell population.

Experiment 2 was performed to further explore the effect of peripherally manipulated estradiol concentrations on stress-induced HPA axis hormone induction. Reports have suggested that estrous cycle stage influences on HPA axis responses to
acute stress are due to the augmenting effect of estrogen specifically on this system, as opposed to other circulating sex hormones such as progesterone (Viau & Meaney 1991; McCormick et al., 2002; Lunga & Herbert 2004; Figueiredo et al., 2007; Larkin et al., 2010). It is possible that in Experiment 1 we did not see an effect of the estrous cycle because all of our manipulations were performed in the morning, when estradiol concentrations in proestrus females are still rising. Therefore in this experiment we targeted higher concentrations of this hormone, but still occurring within the physiological range (Bridges & Byrnes, 2006; Strom et al., 2008). However, no augmenting effect of estradiol following ovariectomy on HPA axis hormone concentrations after restraint stress was observed. On the contrary, the opposite effect was observed, namely reducing estradiol concentrations via ovariectomy did not decrease basal, and significantly increased restraint stress-induced, ACTH concentrations following this manipulation. In fact, at least one study has demonstrated an inhibitory effect of either estradiol alone, or estradiol in conjunction with progesterone administration on restraint stress-induced ACTH secretion (Young et al., 2001), and this, along with our data, conflicts with other published reports (Viau & Meaney 1991; 2004). It is important to note that although the concentration of estradiol in OVX + E₂ females produced by silastic implants is similar in magnitude to those that can occur on the afternoon of proestrus (Bridges & Byrnes, 2006; Strom et al., 2006), the females in Experiment 2 were given constant exposure of the normally fluctuating hormone estradiol (OVX + E₂ group; Figure 2.6) and importantly only after a prolonged period of hormone deprecation. It is possible that activational effects of estradiol can only be seen in the context of normal underlying background levels of estradiol, and possibly
also progesterone, and this idea has been suggested by others (Viau & Meaney, 2004). This theory could explain the seemingly contradictory results presented here in addition to other studies utilizing chronic exposure to a normally cyclical sex steroid after an extended washout period where the animal has no sex steroid exposure whatsoever. For example, estradiol administered in this way suppresses both stress-induced HPA axis hormone induction and Fos labeling in the PVN (Dayas et al, 2000), and even PVN CRF mRNA expression is decreased by such estradiol treatment (Grino et al, 1995). However, the data from intact females presented here still provide no evidence for augmenting effects of estrogen on central HPA axis neurocircuitry following acute stress in even normally cycling animals. This leads to the conclusion that circulating sex steroids in females may not always play a significant role in the magnitude of the HPA axis response to psychological stressors.

Another intriguing possibility is that the nature and magnitude of sex differences in stress-induced HPA axis response may depend on stressor modality. For example, Iwasaki-Sekino and colleagues (2009) observed higher basal and footshock stress-induced levels of CRF mRNA in the female PVN and CeA, whereas Viau and colleagues (2005) observed higher basal and restraint stress-induced CRF mRNA expression in the male CeA, and only a higher basal, but not stress-induced, level of PVN CRF expression in females following this stressor. Another study saw no significant effect of sex on c-fos mRNA expression in the PVN, LS, or MeA 30 min after the termination of the same length of restraint stress (Figueiredo et al, 2002). It is possible that different types of stressors, or even different lab procedures for the same stressor could result in differential sex differences in HPA axis responses, and a more
whole brain wide analysis of stress-induced activation may aid in understanding the nature of sex effects on stress responses in general.

In summary, these results demonstrate that HPA axis responses to acute restraint stress are affected by sex in a complex way, and that regions outside of the PVN should be considered when exploring sex differences in brain activation following acute stress. These results indicate that following restraint, similar stress responsive neurocircuitry is activated in the female compared to male brain, although the magnitude of this activation in certain brain regions is sex specific, despite displaying similar somatosensory processing. Females were found to have more CRF-producing and activated neurons in three brain regions: the BSTav, the MPOA, and PVN, areas known to be involved in the perception of threat and that are likely candidates for sex-specific stress responses, and that increased activation in the female PVN occurs specifically in this population. Furthermore, this study suggests that changes across the estrous cycle do not significantly and consistently contribute to the magnitude of HPA axis responses to restraint stress.
CHAPTER THREE:

STRESSOR-SPECIFIC EFFECTS OF SEX ON HPA AXIS HORMONES AND ACTIVATION OF STRESS-RELATED NEUROCIRCUITRY

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Abstract

Experiencing stress can be physically and psychologically debilitating to an organism. Women have a higher prevalence of some stress-related mental illnesses, the reasons for which are unknown. These experiments explore differential HPA axis hormone release in male and female rats following acute stress. Female rats had a similar threshold of HPA axis hormone release following low intensity noise stress as male rats. Sex did not affect the acute release, or the return of HPA axis hormones to baseline following moderate intensity noise stress. Sensitive indices of auditory functioning obtained by modulation of the acoustic startle reflex by weak pre-pulses did not reveal any sexual dimorphism. Furthermore, male and female rats exhibited similar c-fos mRNA expression in the brain following noise stress, including several sex-influenced stress-related regions. HPA axis release to noise stress in female rats was not affected by stage of estrous cycle, and ovariectomy significantly increased hormone release. Direct comparison of HPA axis hormone release to two different stressors in the same animals revealed that although female rats exhibit robustly higher HPA axis hormone release after restraint stress, the same effect was not observed following moderate and high intensity loud noise stress. Finally, the differential effect of sex on HPA axis responses to noise and restraint stress cannot readily be explained by differential social cues or general pain processing. These studies suggest the effect of sex on acute stress-induced HPA axis hormone activity is highly dependent on the type of stressor.
Introduction

For many people, the experience of stress can be debilitating, and can have several negative consequences such as susceptibility to physical ailments like the common cold, but can also predispose one to various psychiatric illnesses. Some of these stress-influenced disorders, such as major depression, generalized anxiety disorder and PTSD, are approximately two times more prevalent in women than in men (Linzer et al 1996; Stein et al, 2000; Kessler 2005; Tolin & Foa 2006; Bekker & Mens-Verhulst 2007; Olff 2007; Vesga-Lopez 2008). The hypothalamic-pituitary-adrenal (HPA) axis is one of many systems activated by the experience of physical and psychological threats. Dysregulation of the HPA axis has been linked to several stress-related psychopathologies in humans (Ehlert et al, 2001), so understanding how sex can affect this system may lead to insight into female susceptibility to these disorders. Since rodents share the majority of both central and peripheral components of the HPA axis, the present studies utilize male and female rats to investigate the effect of sex on the two main hormone regulators of the HPA axis: adrenocorticotropic releasing hormone (ACTH), and corticosterone (CORT), as well as activation of stress-related brain regions.

Female rats have been reported to have very different responses to stressful stimuli than males. For example, female and male rats have been shown to exhibit different behaviors (Albonetti & Farabollini 1995), physiological responses (Carter et al, 1986; Elliott et al, 2003; Nickerson et al, 2006; Uji et al, 2007), and even nociceptive responses (Aloisi et al, 1994; Gamaro et al, 1998) following a stressful experience. In addition to these measures, it has been clear for many years that female rats have
markedly different HPA axis profiles than males (Kitay 1961; 1963). Specifically, female rats exhibit higher basal resting levels of both ACTH and CORT (Critchlow et al 1963; Heinsbroek et al, 1991). Female rats also display significantly higher stress-induced release of HPA axis hormones following a variety of stressors, including footshock (Heinsbroek et al, 1991; Rivier 1999), neonatal handling (Panagiotaropoulous et al 2004), restraint (Le Mevel et al, 1978; 1979; Aloisi et al 1994; 1998; Viau et al, 2005; Babb et al 2013), and immune challenge (Seale et al 2004a, 2004b). The present studies utilize loud noise as a psychological stressor, which has several advantages over the aforementioned stimuli. First, the neurocircuitry involved in the sensory processing of audiogenic stimuli is well characterized, and largely does not overlap with stress-activated neurocircuitry. In addition, the intensity of the noise stimulus (or the loudness, measured in decibels) can be manipulated. Furthermore, we have previously characterized the effect of a single 30 minute audiogenic stressor in male rats demonstrating a graded release of HPA axis hormones in response to increasing intensities of loud noise, and a complimentary increase in activation of stress-related neurocircuitry (Burow et al, 2005).

The first experiment presented here tested whether female rats exhibit augmented release of HPA axis hormones to very low intensity noise exposure compared to male rats, indicating a lower threshold of stress responsiveness in female compared to male rats. Experiment 2 explored whether females display more prolonged HPA axis hormone release to a higher, but still moderate, intensity noise exposure compared to males, and whether this leads to sex differences in stress-related neurocircuitry after this stressor as we have previously reported following restraint
stress (Babb et al, 2013). Because of the unexpected lack of sex differences on HPA axis responses to noise stress in the first 2 experiments, the next experiment tested whether equivalent HPA axis responses after noise stress were due to sex differences in auditory system functioning using modulation of acoustic startle reflex (Experiment 3). Next the effect of circulating sex steroids on HPA axis hormone release in female rats was tested by comparing HPA axis hormone release to a high intensity loud noise stressor in intact male and female rats, and ovariectomized female rats with or without estrogen replacement (Experiment 4). Experiment 5 directly compared HPA axis hormone release to both restraint and moderate and high intensity noise stress in male and female rats to investigate whether the effect of sex on these measures is dependent on the type of stressor. Restraint and noise exposure differ in two ways that could potentially be the cause of the observed differential effect of sex. Namely, restraint is presented to rats in groups (of both sexes) simultaneously, allowing for the sight, sound, and smell of other rats being stressed, whereas noise is presented in isolated sound-attenuating chambers. Also, restraint typically is preceded by a basal blood sample, whereas noise is not. Therefore it is possible that female rats could be responding more to either of these two factors, leading to the robust effect of sex that is observed after restraint but not noise. Thus, the last experiment (Experiment 6) tested whether the social context of the stressor, or the effect of pain due to blood sampling could influence the effect of sex on HPA axis hormone release to restraint stress. Together, these data suggest that sex differences in stress-induced HPA axis hormone release and brain activation of stress-related neurocircuitry are highly dependent on stressor modality.
Materials and Methods

Subjects

Young adult (2-5 m.o.) female and male Sprague-Dawley rats weighing 200-225g at the time of arrival were used for all experiments, and were obtained from either Harlan Laboratories (Indianapolis, IN, USA) or Charles River (Portage, MI, USA). All animals were allowed to acclimate to the animal facility for one week prior to any experimental manipulation. All animals were housed in clear polycarbonate cages either in same-sex pairs or groups of four with standard wood shavings bedding and *ad libitum* access to food (standard rat chow) and water. All colony rooms were maintained on a controlled 12:12 light:dark cycle (lights on at 7am) under constant temperature and humidity. To coincide with the nadir of the diurnal rhythms of both ACTH and CORT, all experimental procedures were performed between 9:00 am and 12:00 pm. Because of the large number of animals used in each study and in order to perform experiments on females in a particular stage of estrous cycle, all studies were performed across several days. Two to eight animals were run on any given day for each experiment, and groups were equally represented across days. Care was taken to always run at least one male each day of an experiment for all experiments. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the National Research Council’s (1986) *Guide for the Care and Use of Laboratory Animals*.

Estrous Cycle Verification
Prior to any stress presentation, all female rats were monitored daily for estrous cyclicity via vaginal lavage samples examined with light microscopy. Only females displaying at least two full normal 4-5 day estrous cycles were used in each study. Males in all experiments were handled daily to control for this procedure. On each day of stress presentation, the predicted estrous cycle stage was verified immediately following the stressor. In experiments targeting proestrus specifically, any female not in proestrus was eliminated from statistical analysis.

**Stress apparatus**

The sound-attenuating acoustic chambers consisted of ventilated double wooden (plywood board) chambers, with the outer chamber lined internally with 2.54 cm insulation (Celotex, Suffolk, England). The internal dimensions of the inner box were 60 cm (w) x 38 cm (d) x 38 cm (h), within which a rat home cage could be placed. The middle of the ceiling of each inner chamber was fitted with a single 6 x 9 inch Optimus speaker (Radio Shack, #12-1769-120 W RMS). Light within the inner chamber was provided by a florescent bulb (15W) covered with red tissue paper in the upper left corner. Two fans (upper left and lower right corners) allowed fresh air to flow into the inner chamber while also removing stale air. Noise was produced by a General Radio (#1381, Concord, MA) solid-state random noise generator with the bandwidth set at 2 Hz-50 kHz. The output of the noise generator was amplified (Pyramid Studio Pro #PA-600x, Brooklyn, NY) and delivered to each speaker. The speaker characteristics allowed sound delivery between 20 and 27,000 Hz, rolling off quickly (20 dB/octave) at both ends. Noise intensity was measured directly under the speaker on the bottom of the inner chamber with a Sound Level Meter (A scale, #33-2050, Radio Shack). The
ambient noise level inside the chamber was approximately 60 dB (background noise level), which was approximately 5 dB above the ambient noise level in the colony rooms due to the noise from the air circulating fans within each chamber. Unless otherwise stated, the entire home cage of each animal (singly housed with food and water) was placed into the noise stress apparatus the afternoon prior to noise exposure. Noise exposure given the following morning then allows for the measurement of HPA axis hormone increase that occurs solely due to the noise itself, rather than from the home cage of the animal being moved from the colony room to the testing apparatus.

Restraint was conducted in clear ventilated, cylindrical Plexiglas tubes of adjustable length. The size of the tube (23.5 cm in length and 7 cm in diameter for the males; 15.5 cm long and 6.3 cm diameter for the females) restricted movement in all directions with the rat’s tail protruding from the cylinder, but did not interfere with breathing.

**Acoustic Startle Testing**

Startle testing was performed as the authors have previously described (Masini et al 2008; 2012). Briefly, startle testing occurred in eight identical ventilated isolation chambers (Hamilton-Kinder LLC, San Diego, CA), and was illuminated by 8W bulbs located in the ceiling of the chambers. Each startle chamber contained a rat holder (19.05×9.14×10.67 cm acrylic cage) held in place on a platform load cell that detects rats’ movements. Startle amplitude was defined as the maximum load cell output (amplified and digitized) during the first 200 ms after the startle stimulus onset. The baseline startle stimuli (pulses) were 50 ms (rise-decay: 1 ms) bursts of white noise at
intensities of 105 dBA (sound pressure level, SPL; A scale). Background noise (white noise) of 60 dBA was delivered throughout the startle test. The auditory gap stimuli consisted of a 4, 8, or 16 ms gap in the background white noise (reduction of noise from 60 to 52 dBA, which was the ambient noise intensity in the acoustic chamber) presented 75 ms before the pulse stimuli (GAP trials). Facilitation of the acoustic startle response (pre-pulse facilitation; PPF), was induced with noise increment stimuli consisting of bursts of white noise (rise–decay: 1 ms) at intensities of 62, 64, or 68 dBA, presented 10 ms before the pulse and ending with the presentation of the startle pulses (PPF trials). Pre-pulse inhibition (PPI) of the acoustic startle response was induced with auditory prepulse stimuli consisting of 20 ms bursts of white noise (rise–decay: 1 ms) at intensities of 63, 66, or 70 dB, presented 50 ms before the pulse stimuli (auditory PPI trials). A 400-lux, 20-ms flash of light, provided by arrays of 6 × 20 diodes on each side of the cage (rise–decay: 100 µs) each located 3 cm from the cage and extending the entire height of the cage, served as a visual PPI stimulus presented 50 ms before the pulse (Light PPI trials). For the startle test, the rats were placed in the holders, and after a 5 min acclimation period, the first of 20, 105 dBA pulses were presented at a fixed 20-s interstimulus interval (ISI). These initial startle pulses produce some startle habituation, which reduce variability and stabilize baseline startle amplitudes, but they were not used in the estimation of startle modification. Twenty seconds later, the first of 100 additional test trials were presented, with a fixed ISI of 20 s, consisting of pulse alone stimuli (20 trials), intermixed with GAP (10 trials at each gap duration), PPF (10 trials at each prepulse increment intensity level), or PPI trials (10 trials at each prepulse
intensity and visual stimulus level). There were 10 occurrences of each trial type in a quasirandom sequence.

**Blood collection**

For tail blood collection, animals were gently held immobile using a clean towel, and a small incision was made in a lateral tail vein with the corner of a razor blade. Blood was collected using heparinized hematocrit capillaries deposited into ice-chilled 0.5mL microcentrifuge tubes (for CORT assays), and using uncoated hematocrit capillaries deposited into similar tubes containing EDTA (20 mg/ml) for ACTH assays. This procedure lasted less than 4 min to prevent detection of elevated hormone levels due to the blood collection procedure itself. These blood samples were centrifuged at 14,000 rpm at room temperature for 1 min. After the final stressor exposure, rats were decapitated and trunk blood was collected into large EDTA-coated tubes (Vacutainers). These tubes were then centrifuged at 2,000 rpm for 10 min at 4°C. For all samples, plasma was then pipetted into 0.5ml Eppendorf microcentrifuge tubes and stored at -80°C until assayed for hormone concentrations.

**Experiment 1: Effect of sex on HPA axis responses to low intensity noise**

To test whether sex affects the threshold at which noise stress can activate the HPA axis, male and female (in either diestrus, proestrus, or estrus) rats were exposed to 30 minutes of either 60 (no noise control; male: n = 5; female: n = 26), 75 (male: n = 5; female: n = 19), or 80 (male: n = 5; female: n = 30) dB white noise. These noise intensity levels were chosen based on previous studies using noise in male rats (Burow et al, 2005). Immediately following the 30 min noise exposure, animals were sacrificed,
trunk blood was collected and plasma concentrations of ACTH and CORT were determined.

**Experiment 2: Effect of sex on HPA axis hormone responses to moderate intensity noise and activation of sensory and stress-related brain regions**

To determine whether females exhibit a higher acute, and/or more prolonged HPA axis response following the termination of a moderate intensity of noise stress, male and proestrus female rats (n = 7-9/group) were exposed to 90 dB noise for 30 minutes and were sacrificed either immediately following noise exposure (30 min), or 15, 30, or 60 minutes after cessation of the noise (45, 60, and 90 min groups, respectively). This noise intensity consistently produces a significant increase in HPA axis hormones in male rats (Burow et al, 2005). In this experiment, only females predicted to be in proestrus were given stressor exposure due to the prior finding that this stage of estrus induces significantly higher HPA axis responses to stress than any other stage (Viau & Meaney, 1991). Additional male and proestrus female rats were sacrificed immediately the morning of the experiment to serve as no stress controls. Plasma from all animals was analyzed for concentrations of ACTH and CORT, and brains from no stress controls (0 min time point) and animals sacrificed immediately upon termination of the stressor (30 min time point) were analyzed for c-fos mRNA using in situ hybridization. Brain regions analyzed were chosen either due to their involvement in the sensory perception of noise (Campeau & Watson, 1997; Burow et al, 2005), or because they have previously been reported to be sensitive to a variety of stress situations.
Experiment 3: Effect of sex on auditory system functioning via acoustic startle responses

In order to test whether female rats did not display higher magnitude HPA axis responses to noise as expected due to potential sex differences in hearing thresholds, male (n = 8) and female (n = 13) rats were given acoustic startle testing daily for 5 days in order to have each female tested in each day of the estrous cycle, since both menstrual cycle (Jovanovic et al, 2004) and ovarian steroid manipulation in rodents (Charitidi et al, 2012) have been documented to affect acoustic startle reflexes. Rats were run in same-sex groups, and the order of testing of these groups was alternated each day. The acoustic startle testing included trials for gap detection (which should inhibit the normal startle response), pre pulse facilitation (PPF) of the acoustic startle response by increment pulses (quieter pulses immediately preceding the startle pulse), and pre-pulse inhibition (PPI) of the startle response by an auditory or visual pre-pulse. These three different trial types were included on the basis that disruptions of these different prepulse manipulations are linked to different brain regions involved in auditory perception (Ison et al, 1991; Kelly et al, 1996; Bowen et al, 2003), and are routinely used to test auditory threshold shifts (Parham et al, 1988; Willott et al, 1994). The visual PPI trial type was included to serve as a non-auditory cue control. One to two cycles later, the same animals (male and females in proestrus) were exposed to 30 min of 80 dB noise, following which animals were sacrificed and plasma CORT concentrations were determined. Again, proestrus was targeted as the stage of estrous cycle due to this stage likely having the largest magnitude HPA axis responses to stress (Viau et al, 1991).
Experiment 4: Effect of sex and ovariectomy with or without estrogen replacement on HPA axis responses to high intensity noise

For this experiment only, male and female rats (Sprague-Dawley originally obtained from Harlan Laboratories) were raised in the animal colony at the University of Colorado at Boulder. When they were approximately 3 months of age, females were ovariectomized via bilateral incisions and implanted with silastic capsules as described previously (Strom et al, 2008). Briefly, 30 mm long silastic tubing segments (Inner/Outer Diameter: 1.575/3.173 mm, Dow Corning, Fisher Scientific Inc, USA) were filled with 70 µl of either sesame oil vehicle (OVX; n = 10) or 17β-estradiol (E₂; 180µg/ml; Sigma) in sesame oil (OVX + E₂; n = 10), and were sealed with 5 mm long segments of 2 mm diameter wooden applicator sticks. Other female (intact females; n = 12) and male (intact males; n = 10) rats received sham surgery on the same days: identical bilateral incisions were made into the muscle walls and immediately sutured without removal of any tissues. Animals were given one week to recover from surgery prior to further experimental manipulation. All rats were then habituated to being transported down the hallway from the colony room to a behavioral testing suite for 5 consecutive days to reduce HPA axis hormone increases due to this manipulation. Basal blood samples were taken outside the colony room on the second day of this habituation (prior to being transported to the testing suite). The morning after the 5th day of habituation to being moved, all rats were brought to the testing suite and remained untouched for 15 min, and were then exposed to 30 min of 105 dB noise stress, immediately after which a blood sample was taken for plasma determination of ACTH and CORT. Intact females were monitored for stage of estrous cycle, but no
specific stage was targeted. Forty-eight hours later, all animals were again transported from the colony room to the behavioral testing suite and left untouched for at least 15 min, but were then exposed to 30 min of restraint (these data have been previously published, as well as estradiol concentrations of all animals at baseline and after restraint; see Babb et al, 2013).

Experiment 5: Effect of sex on HPA axis responses to restraint and noise stress in the same male and proestrus female rats

In the previous experiment, one group of sham-operated intact females was compared directly to male rats also receiving sham surgery. However, the estrous cycle of these female sham-operated intact females was not controlled. It is possible that had the proestrus cycle stage been targeted in the intact females in this study, higher HPA axis hormone concentrations to high intensity noise in proestrus female compared to male rats may have been observed. Therefore this experiment tested whether male (n = 27) and female rats in proestrus (n = 27) would exhibit still similar HPA axis responses to moderate or high intensity noise stress, even after demonstration of a robust sex difference following an initial restraint exposure. On the afternoon before the first stressor (restraint), male and diestrus female rats were transported to a room adjacent to where stress occurred. This room was empty except for these animals, and was identical to the main colony room in terms of light cycle, temperature, and humidity conditions. The following morning, all animals were exposed to 30 minutes of restraint, male and female rats alternated, and all rats staggered by 5 min such that several animals were being restrained on the same bench at the same time, after which they were returned to their home cages. Blood was collected in the same room via tail nicks...
from all animals just prior to, and immediately after, restraint, as well as 30 minutes after returning to their home cage (0, 30, and 60 min time points, respectively). Following the final tail nick at 60min, stage of estrous cycle (proestrus) was verified for all the females. One estrous cycle later for the females, the same animals (male and female) were exposed to 30 minutes of 80 dB noise, and blood was again collected from a tail vein. Two to three weeks later the same males and females (again in proestrus) were then exposed to 30 minutes of 105 dB noise, and were sacrificed immediately for collection of trunk blood and plasma determination of ACTH and CORT concentrations.

*Experiment 6: Influence of social context and blood sampling on the effect of sex on HPA axis responses*

To test the hypothesis that female rats display higher HPA axis responses to restraint but not noise due to the social context of the stressor (noise was always presented to rats in isolated sound-attenuating chambers; restraint was always presented in groups where all animals can potentially hear, see, and smell other rats also being stressed), male and female rats on each test day were exposed to either restraint in a group setting or in isolation (n = 7-9/group). Our hypothesis was that female rats may have exhibited higher HPA axis responses to restraint stress than male rats because female rats were responding more to the sight, smell, and/or sound of other rats being stressed around them more than male rats, rather than just to the experience of the restraint itself. This hypothesis would then explain why no effect of sex on HPA axis hormone responses to noise is observed, since this stressor is presented to isolated rats. Treatment conditions were alternated each day. Animals in both treatment conditions were staggered by 5 minutes to allow time for baseline blood
collection prior to the start of stress, and 4-6 animals were stressed on each day. On the morning of restraint, a baseline blood sample was taken via tail nick from males and females in proestrus. Animals were then placed in a restraint tube, and then were placed either in one open sound-attenuating chamber normally used for noise stress exposure next to other animals also being restrained, or one animal was placed in one of these chambers, and the doors were closed to isolate each animal inside for the duration of the restraint stress. All animals were restrained for 30 min each, and blood was collected immediately following the end of the restraint via tail nick, and the animal was returned to its home cage. The home cages of the group stressed animals were left in the same room as restraint occurred, and all blood collections were also performed in this room, so animals being stressed in groups were exposed not only to the sounds, smells, etc from other rats also being stressed, but also of other rats having blood samples taken. The home cages of the animals stressed in isolation were in a separate adjacent room, and all blood collections were done in a third room, so none of these animals were exposed to the cues of other animals being stressed. A final blood sample was taken again via tail nick 30 minutes after returning the animals to their home cages. After the final blood sampling, stage of estrous cycle was verified in the females and males were handled. Then all animals were returned to the main colony.

The animals remained in the main colony experimentally untouched for ~2 weeks. Then to test whether female rats display higher magnitude HPA axis responses to restraint stress due to the initial baseline blood sample that is typically taken prior to presentation of this stress, the same males and females (again in proestrus) were placed in restraint tubes for 30 minutes and a single blood collection via tail nick was
taken either immediately after the cessation of the stressor, or 30 minutes after returning to their home cage (n = 7-9/group). All animals were restrained in a group setting, and all animals remained in the restraint room until all the tail nicks were done for each day. The animals were then returned to the main animal colony. Blood samples from both experiments were then analyzed for plasma ACTH and CORT concentrations.

**ACTH IRMA**

Plasma ACTH concentrations were assayed using a commercially available immunoradiometric assay kit (Cat # 27130; DiaSorin, Stillwater, MN, USA); which has a sensitivity of 1.5 pg/ml, and inter- and intra-assay coefficients of ~4.6% and ~ 4.18%, respectively. This IRMA kit recognizes 100% of whole molecule ACTH (detects all 39 amino acids), and as such is very specific, with undetectable cross-reactivity for α- and β-MSH, as well as β-Endorphin. The assay was performed according to the kit directions. Briefly, 200 µl of plasma or plasma diluted with zero standard (provided with the kit) was incubated overnight with a ¹²⁵I-labelled monoclonal antibody specific for ACTH amino acids 1-17, a goat polyclonal antibody specific for ACTH amino acids 26-39, and a polystyrene bead coated with a mouse anti-goat IgG. Only ACTH in the sample with all 39 amino acids bound both antibodies to form an antibody complex on the polystyrene bead. The following day, tubes containing the radioactive beads were washed to remove any unbound reagents and the amount of radioactivity still left on each bead was determined with a gamma counter. The concentration of ACTH for each sample was calculated by fitting values for each sample into a standard curve of known ACTH dilutions processed concurrently.
**CORT ELISA**

Plasma CORT concentrations were analyzed using a commercially available kit (Cat. # 901-097; Assay Designs, Ann Arbor, MI, USA). This is a highly specific assay (100% cross-reactivity with CORT, 28.6% with deoxycorticosterone, and 1.7% with progesterone), with a sensitivity of 26.99 pg/ml, and inter-/intra-assay variability of ~7.5% and ~9.7%, respectively. The kit directions were followed with the exception of a modification to use a smaller volume of plasma as follows. The steroid displacement reagent (0.5 µl/ml; provided with the kit) was added to the assay buffer. Ten µl of plasma was then diluted 1:50 with the amended assay buffer. The diluted plasma samples were then processed according to the kit directions. This method was found to result in assayed CORT levels equivalent to the method in the kit directions (data not shown). Briefly, plasma samples and diluted CORT standards were incubated in a 96-well plate coated with anti-sheep antibody raised in donkey, together with CORT with a covalently attached alkaline phosphatase molecule, and a sheep polyclonal antibody for CORT. After a 2 hr incubation period the plate was washed to remove all unbound reagents and a substrate was added. After 1 hr the color reaction was stopped and the intensity of color in each well was analyzed at 405 nm using a microplate reader (Biotek EL808; Winooski, VT, USA). The concentration of CORT for each sample was calculated by fitting unknown values into a standard curve of known CORT dilutions processed concurrently.

**In Situ Hybridization**
The method for *in situ* hybridization has been described previously (Day et al, 2005). Briefly, whole brains stored at -80°C were mounted on chucks at -20°C. Ten micrometer thick sections were cut on a cryostat (Model 1850; Leica Microsystems, Buffalo Grove, IL, USA), thaw-mounted onto polylysine-coated slides and stored at -80°C. Slides were taken from storage and immediately fixed in cold 4% paraformaldehyde for 1 hr, acetylated in 0.1M triethanolamine (pH 8.0) with 0.25% acetic anhydride for 10 min, dehydrated through graded ethanol to 100% and air dried. A riboprobe against c-*fos* mRNA (680 mer; courtesy of Dr. T. Curran, St. Jude Children’s Hospital, Memphis, TN) was generated using standard transcriptional methods and labeled with $^{35}$S-UTP (Perkin Elmer, Waltham, MA). Brain sections were hybridized overnight at 55°C with the riboprobe diluted to 1-3 x10$^6$ c.p.m. per 70 µl in hybridization buffer containing 50% formamide, 10% dextran sulfate, 3X saline sodium citrate, 50mM sodium phosphate (pH 7.4), 1X Denhardt’s solution, 0.1 mg/ml yeast tRNA, and 10mM dithiothreitol. The following day, the probe was washed from the slides, and they were then treated with RNase A (200 µg/ml; pH 8.0) at 37°C for 1 hr, washed to a final stringency of 0.1X saline sodium citrate for 1 hr at 65°C, dehydrated again in graded ethanol to 100%, and air dried. Slides were then exposed to X-ray film (BioMax-MR; Eastman Kodak, Rochester, NY) for 7-12 days. Films were then analyzed as described below.

Levels of c-*fos* mRNA were analyzed by computer-assisted optical densitometry by an experimenter blind to the treatment conditions. Images of each individual brain section were captured digitally (CCD camera, model XC-77; Sony, Toyko, Japan), and digital images were then analyzed using Scion Image (Version 4.03 for Windows; ScionCorp).
First, the relative optical density of the x-ray film was determined using a macro within Scion Image (written by Dr. S. Campeau) which allowed the automatic determination of a signal above background. Specifically, for each section, a background sample was taken over an area of white matter, and the signal threshold was set as 3.5 standard deviations above the mean gray value of the background. The remaining pixels above this threshold were then analyzed within the brain region of interest. It should be noted that although background criteria were relatively stringent, this method still results in a few pixels above background in areas on the x-ray film not on the brain section, indicating that even weak intensity mRNA signal on the tissue is detected. For consistency, a different template was created for each brain region, and was placed using anatomical landmarks based on the white matter distribution of the unstained tissue, according to a standard rat brain atlas (Paxinos & Watson, 2005). The number of pixels above background was multiplied by the signal above background to give an integrated density value for both hemispheres throughout the rostral-caudal extent of each brain region of interest. This method has been reported to reflect both the number of cells expressing mRNA and the expression level per cell, as determined by cell and grain counts of emulsion-dipped slides (Day et al, 2005). The mean integrated density for each animal was then calculated by averaging the highest 2 or 3 values for each hemisphere depending on the brain region resulting in a single value for each animal representing the peak of c-fos mRNA expression for each brain region of interest. Standardized scores (z scores + 1.0) were then calculated for each brain region to allow for the display of many brain regions with very different mRNA expression levels on the same graph.
**Statistical Analysis**

All hormone data were analyzed with appropriate one-, two-, or three-way ANOVAs (and with repeated measures when appropriate), with significance set at $p = 0.05$. Significant main effects in repeated measures ANOVAs were explored further with one-way ANOVAs. Further post-hoc analyses, when necessary, were performed using independent samples t-tests. To test for a sex difference in c-fos mRNA expression levels across brain regions, data were analyzed using independent samples t-tests, with statistical significance for these tests set at $p < 0.01$ to reduce the possibility of Type I error. SPSS Statistics (version 18 for Windows) was used for all statistical analyses.

**Results**

*Experiment 1: Effect of sex on HPA axis responses to low intensity noise*

Figure 3.1 demonstrates the effect of noise at low intensities and sex on HPA axis hormone secretion. There was no effect of stage of estrous cycle on either ACTH or CORT secretion at any of the intensities analyzed ($p$'s > 0.05), so the female data were pooled for further analysis. Noise induced a significant activation of the HPA axis in all animals overall, as seen in a two-way ANOVA by a main effect of noise intensity on both ACTH ($F_{2,89} = 6.57, p < 0.01$; Fig 3.1A) and CORT ($F_{2,89} = 7.80, p < 0.01$; Fig 3.1B) concentrations. Post hoc analyses revealed that ACTH concentrations after 80 dB noise were significantly higher than at 60 dB (controls) ($p$'s < 0.05), and that CORT concentrations after 80 dB noise were significantly higher than at 60 and 75 dB ($p$'s < 0.05). Overall, males and females did not show different thresholds of noise intensity to
Figure 3.1

The effect of sex on ACTH (A) and CORT (B) concentrations at baseline (60 dB) and after 30 min of low-intensity noise stress (75 and 80 dB) is displayed in Figure 1. All values are displayed as means ± 1 SEM. *: $p < 0.05$ compared to male rats at the same intensity. #: $p < 0.05$ compared to baseline (60 dB), regardless of sex. ^: $p < 0.05$ compared to 75 dB noise, regardless of sex.

activate the HPA axis. Although there was no main effect of sex on either ACTH ($F_{1,89} = 3.39$, $p = 0.069$) or CORT ($F_{1,89} = 0.85$, $p = 0.36$), specific analysis of controls demonstrated that female rats had higher basal levels of ACTH ($t_{22} = 2.07$, $p < 0.05$), and a trend towards significantly higher CORT levels ($t_{22} = 1.71$, $p = 0.06$) at 60 dB (no
noise controls) compared to male rats. There was not a significant interaction between sex and noise intensity ($p > 0.05$).

**Experiment 2: Effect of sex on HPA axis hormone responses to moderate intensity noise and activation of sensory and stress-related brain regions**

Figure 3.2A shows the effect of sex on ACTH responses to noise across time. Thirty minutes of 90 dB noise resulted in a significant increase in ACTH concentration overall in both males and proestrus females, as seen by a significant main effect of time on ACTH concentration ($F_{4, 71} = 17.119$, $p < 0.001$). Post hoc tests revealed that ACTH levels were significantly higher at 30 min than at all other time points ($p < 0.001$), and that by 45 min ACTH levels were back down near baseline levels for both sexes ($p's > 0.05$). No other time points for ACTH concentrations differed significantly from each other. Overall, there was no main effect of sex on ACTH secretion. However, there was a sex by time interaction ($F_{4, 71} = 2.594$, $p = 0.044$), and post hoc tests revealed that this was due to male rats having a higher concentration of ACTH at 30 minutes as compared to female rats ($p < 0.05$).

Figure 3.2B shows the effect of sex on CORT responses to 30 minutes of 90 dB white noise. Overall, noise also significantly increased CORT concentrations regardless of sex ($F_{1, 71} = 14.433$, $p < 0.001$). Post hoc tests revealed that CORT concentrations at 30 minutes were significantly higher than all other time points except for 45 minutes ($p's < 0.05$). At 45 minutes, CORT levels were still significantly higher than at 90 minutes and baseline ($p's < 0.05$). By 60 minutes, CORT concentrations were not significantly different from baseline for both sexes ($p's > 0.05$). There was no main effect of sex,
The effect of sex on the recovery from HPA activation in response to 30 min of 90 dB noise (black bar) is displayed in Figure 3.2. All values are displayed as means ± 1 SEM. *: \( p < 0.05 \) compared to males at that time point. #: \( p < 0.05 \) compared to baseline (0 min) regardless of sex.

A) ACTH levels increased significantly in all animals after 30 min, and were back down to baseline levels by 45 minutes for both sexes. Post-hoc tests for a significant sex by time interaction revealed that males had significantly higher levels of ACTH than females at 30 min \( (p < 0.05) \). B) CORT levels increased significantly for all animals by 30 minutes, and remained elevated until 60 minutes for both sexes. In addition, females had significantly higher baseline levels of CORT than males \( (p < 0.05) \).

however females had significantly higher CORT levels at baseline as compared to males \( (t_{13} = 2.627, p < 0.05) \). There was no sex by time interaction on CORT secretion.

The effect of sex on basal (from 0 min time point) and noise stress-induced (from 30 min time point) c-fos mRNA expression is displayed in Figure 3.3. Noise significantly increased c-fos mRNA expression in the brains of all animals \( (p \text{'s} < 0.01) \). However,
there was no effect of sex in any brain region analyzed at either 0 or 30 min of noise stress ($p$'s $> 0.01$).

**Figure 3.3**

The effect of sex on relative c-fos mRNA expression at baseline and following 30 min of noise stress in stress-related (PL, IL, LS, BSTav, MPOA, DMH, PVN, MeA, BLA) and auditory processing (Aud Ctx, MGN, IC, VCA) brain regions is displayed in Figure 3.3. All values are displayed as mean standard score + 1 (in arbitrary units; all standard errors are equal to 1). There was no effect of sex on either basal or stress-induced c-fos expression in any brain region examined. Abbreviations: PL: prelimbic cortex; IL: infralimbic cortex; LS: lateral septum; BSTav: bed nucleus of the stria terminalis, anteroventral subdivision; MPOA: medial preoptic area; DMH: dorsomedial nucleus of the hypothalamus; PVN: paraventricular nucleus of the hypothalamus; MeA: medial nucleus of the amygdala; BLA: basolateral nucleus of the amygdala; Aud Ctx: auditory cortex; MGN: medial geniculate nucleus of the thalamus; IC: inferior colliculus; VCA: ventral cochlear nucleus.

**Experiment 3: Effect of sex on auditory system functioning via acoustic startle responses**

The effect of sex on various types of pre-pulse manipulations of the startle response (GAP detection, PPF, or PPI), baseline startle amplitude, and CORT response to 30 min of 80 dB noise is displayed in figure 3.4. Each female was in each of the 4
Figure 3.4

The effect of sex on pre-pulse suppression (GAP and PPI) and facilitation (PPF) of the acoustic startle response. All values are displayed as means ± 1 SEM. A) No effect of sex was observed on any manipulation of the acoustic startle response ($p$'s > 0.05). Values represent means of the average for each animal across 5 days of testing. B) Baseline acoustic startle amplitude (corrected for body weight (BW)) is significantly higher in female rats. Values also represent means of each animal's average across the 5 test days. *: $p < 0.05$ compared to male rats. C) No effect of sex on CORT concentration in response to 30 min of 90 dB noise was observed in the same animals.
stages of the estrous cycle at least once throughout the 5 day testing period. Analyzing data from each day of the estrous cycle once from each female revealed no main effect of the estrous cycle on baseline startle amplitude, or any of the pre-pulse manipulations measured (p's > 0.05; data not shown), so further data analysis compared male to female data regardless of the stage of the estrous cycle. Data from male and female rats were then collapsed across days of testing, so that each data point represents the average response for that stimulus type across the 5 days. Although there was no significant effect of sex on any of the pre-pulse manipulations examined (p's > 0.05; Figure 3.4A), female rats exhibited a significantly higher baseline startle amplitude compared to male rats (t_{19} = 2.09, p = 0.03; Figure 3.4B). However, the same male and female rats (in their next proestrus stage) did not differ in their CORT response to 30 min of 80 dB noise (t_{18} = 2.1, p > 0.05; Figure 3.4C).

*Experiment 4: Effect of sex and ovariectomy with or without estrogen replacement on HPA axis responses to high intensity noise*

The effects of sex and ovariectomy with or without E2 replacement on HPA axis responses to noise stress are displayed in figure 3.5. At baseline, there was no main effect of treatment group on ACTH concentrations (F_{3,38} = 2.40, p=0.08; Figure 3.5A). However, following 30 min of noise stress, there was a significant effect of treatment group (F_{3,38} = 5.39, p=0.003). Specifically, males and OVX females had significantly higher ACTH concentrations than intact females (Sham) and OVX + E2 females (p's < 0.05). Male and OVX female ACTH concentrations did not differ significantly, nor did intact female and OVX + E2 ACTH concentrations. There was a significant main effect of treatment group on basal (F_{3,38} = 3.34, p = 0.03) and stress-induced (F_{3,38} = 4.18, p =
Figure 3.5

The effects of sex and ovariectomy with (OVX + E\textsubscript{2}) or without (OVX) estradiol replacement on HPA axis responses to 30 min of 105 dB noise are displayed in Figure 3.5. All values are displayed as means ± 1 SEM. *: \(p < 0.05\) compared to both intact male and OVX female rats. #: \(p < 0.05\) compared to intact male rats. A) ACTH concentrations before (Basal) and after noise stress. B) CORT concentrations before (Basal) and after noise stress.

0.01) CORT concentrations (Figure 3.5B). Specifically, intact females and OVX + E\textsubscript{2} females had significantly higher basal CORT concentrations than males, and OVX + E\textsubscript{2} females were also significantly higher than OVX females (\(p's < 0.05\)). Male and OVX females, as well as intact and OVX females’ CORT concentrations did not differ significantly (\(p's > 0.05\)) before stress. After noise stress, males had significantly higher CORT concentrations than intact and OVX + E\textsubscript{2} females, and OVX females had
significantly higher CORT concentrations than OVX + E₂ females (p's < 0.05). No other groups' CORT concentrations differed significantly following stress (p's > 0.05).

Experiment 5: Effect of sex on HPA axis responses to restraint and noise stress in the same male and proestrus female rats

The effect of sex on plasma ACTH and CORT concentrations in response to two different acute stressors in the same animals are displayed in figure 3.6. Restraint stress significantly increased ACTH concentrations in all animals, as demonstrated by a main effect of time (F_{2,104} = 147.89, p < 0.001; Figure 3.6A). In addition, females had significantly higher ACTH compared to males overall (F_{1,52} = 35.68, p < 0.001). There was also a significant interaction between time and sex (F_{2,104} = 14.35, p < 0.001); females had a significantly greater increase in ACTH following restraint despite having significantly higher basal ACTH levels. Area under the curve analysis for ACTH concentrations overall confirmed that female ACTH concentrations were significantly higher than in males (t_{52} = 5.88, p < 0.001). Basal and restraint-stress-induced plasma CORT concentrations are displayed in Figure 3.6B. Restraint significantly increased CORT concentrations in all animals (F_{2,104} = 155.63, p < 0.001). Overall, females also had significantly higher CORT concentrations than males (F_{1,52} = 40.43, p < 0.001). In addition, there was a statistically significant interaction between sex and time (F_{2,104} = 15.37, p < 0.001). Restraint led to a significantly greater increase of CORT concentrations at 30 and 60 minutes in female rats despite having already elevated baseline concentrations compared to male rats (p's < 0.001). Area under the curve analysis for CORT concentrations overall confirmed that female CORT concentrations were significantly higher than in males (t_{52} = 6.29, p < 0.001).
Figure 3.6

The effect of sex and type of stress on HPA axis responses in the same male and proestrus female rats (given restraint, then 80 dB noise, then 105 dB noise) is displayed in Figure 3.6. Black bar represents 30 min of restraint stress. *: \( p < 0.05 \) compared to male rats at the same time point. A) Restraint significantly increased ACTH concentrations in all animals, and female rats had significantly higher basal and stress-induced ACTH levels compared to males. B) Restraint also increased CORT concentrations significantly. Females had significantly higher CORT levels at every time point compared to males. C) There were no significant differences in ACTH levels due to either sex or noise intensity. D) No effect of sex or noise intensity on CORT concentrations was observed.

The effect of sex on HPA axis responses to moderate (80 dB) and high (105 dB) intensity noise stress in the same animals is displayed in Figure 3.6C&D. There was no significant effect of sex on ACTH concentration following either 80 dB (\( t_{50} = 1.30, \ p = 0.20 \)) or 105 dB (\( t_{50} = 0.69, \ p = 0.49 \)) noise, as demonstrated in Figure 3.6C. In addition, there was no significant effect of sex on CORT concentration after either 80 dB (\( t_{50} = 1.83, \ p = 0.07 \)) or 105 dB (\( t_{50} = 0.26, \ p = 0.80 \)) noise, as displayed in Figure 3.6D.
Experiment 6: Influence of social context and prior blood sampling on the effect of sex on HPA axis responses

Figure 3.7 displays HPA axis responses to 30 min of restraint given in close proximity with other rats being restrained simultaneously (Group Stressed) or given in isolation (Isolation Stressed) in male and female rats. Plasma ACTH and CORT concentrations were analyzed initially with a 3-way repeated measures design with time (0, 30, and 60 min), sex (Male and Female), and social context (Group or Isolation) as the independent variables. Restraint significantly increased ACTH concentrations in all animals, demonstrated by a main effect of time ($F_{2,56} = 85.73, p < 0.001$; Figure 3.7A). Importantly, exposing females to restraint stress in isolation did not eliminate the sex difference seen in HPA axis responses. Specifically, females had significantly higher levels of ACTH overall, as demonstrated by a main effect of sex ($F_{1,28} = 23.90, p < 0.001$). There was also a trend for a time by sex interaction ($F_{2,56} = 3.00, p = 0.058$), and tests of within-subjects contrasts showed a significant linear fit for this interaction ($F_{1,28} = 4.44, p = 0.044$), meaning that females had a trend for a larger increase in ACTH concentrations after stress (30 and 60 min; see Figure 3.7A). A further analysis of the change in ACTH concentrations from 0 to 30 min revealed a significant main effect of sex ($F_{1,31} = 5.12, p = 0.03$), demonstrating that females had a significantly sharper rise in ACTH concentrations between these time points compared to males, regardless of the social context of the stressor (Group vs. Isolated). Although there was no main effect of social context ($F_{1,28} = 2.53, p = 0.14$), there was a trend for a time by social context interaction ($F_{2,56} = 3.06, p = 0.055$), where animals stressed in isolation, regardless of sex, tended to have higher, rather than lower, ACTH concentrations at 30
min. There was no significant two-way interaction between sex and social context or a significant three-way interaction between time, sex and social context of stress on ACTH concentrations (Figure 3.7A).
Figure 3.7

The effects of sex (Male and Female) and social context of stress (restraint given in Groups vs. in Isolation) on ACTH and CORT concentrations over time (0, 30, and 60 min) are displayed in Figure 3.7. Restraint stress for 30 min (black bar) was administered immediately after basal blood sampling performed at 0 min. A) ACTH concentrations overall were significantly affected by stress exposure ($p < 0.001$) and sex ($p < 0.001$), but not social context ($p = 0.14$). Restraint stress significantly increased ACTH concentrations overall, although to a greater extent in females compared to males. B) CORT concentrations overall were also significantly affected by stress exposure ($p < 0.001$) and by sex ($p = 0.001$), but not social context ($p = 0.82$). Restraint stress significantly increased CORT concentrations, and female rats had significantly higher CORT concentrations compared to males. There was also a significant time by social context interaction, where animals stressed in isolation had a significantly more prolonged response to restraint stress than animals stressed in groups, regardless of sex ($p = 0.002$).

A three-way repeated measures ANOVA also revealed a main effect of time ($F_{2,27} = 239.88$, $p < 0.001$) and of sex ($F_{1,28} = 13.93$, $p = 0.001$) on plasma CORT concentrations (Figure 3.7B). Restraint significantly increased CORT concentration in all animals, and overall female rats had significantly higher CORT concentrations than male rats. Although there was no main effect of social context of the stressor on CORT concentrations ($F_{1,28} = 0.06$, $p = 0.82$), there was a significant time by social context interaction on CORT concentration ($F_{2,56} = 5.86$, $p = 0.005$). Post-hoc tests revealed that this interaction was due to animals restrained in isolation having a more prolonged CORT response to stress ($F_{1,31} = 11.75$, $p = 0.002$). There was not a significant time by sex interaction ($p = 0.20$) or time by sex by social context interaction ($p = 0.20$) on CORT concentrations, meaning that the overall effect of sex did not differ depending on either the time point, or the social context of the stressor (restrained in close proximity or in isolation).

Figure 3.8 shows HPA axis responses to restraint with a single blood sample taken at either 30 minutes (immediately after the stressor) or at 60 minutes (30 minutes
Figure 3.8

The effect of sex on HPA axis responses to 30 minutes of restraint without experience of a basal blood sample is displayed in Figure 3.8. *: $p < 0.05$ compared to female rats at the same time. #: $p < 0.05$ compared to 30 min time point. A) Females had significantly higher ACTH concentrations than males in response to 30 minutes of restraint when one blood sample was taken either immediately after (30 min), or 30 min after being returned to the home cage (60 min). All animals overall had significantly lower ACTH levels when blood samples were taken at 60 min compared to animals that had a blood sample taken immediately following restraint (30 min). B) Females also had significantly higher CORT concentrations than males at both time points, and all animals had significantly lower CORT levels when tested at 60 minutes than animals sampled at 30 min.

after animals were returned to their home cages). Overall, eliminating a baseline blood sample prior to restraint did not eliminate the sex difference in HPA axis activation
previously seen after the experience of this stressor. Thirty minutes of restraint followed by a single tail nick resulted in females having larger HPA axis responses as compared to males at both time points. Overall, females had statistically higher ACTH ($F_{1,25} = 6.221, p < 0.05$) and CORT ($F_{1,25} = 11.339, p < 0.01$) concentrations regardless of the time point. Furthermore, overall all animals had significantly lower ACTH ($F_{1,25} = 10.128, p < 0.01$) and CORT ($F_{1,25} = 10.189, p < 0.01$) concentrations at 60 minutes as compared to 30 minutes regardless of sex. There was no significant sex by time interaction for either hormone.

**Discussion**

The experiments presented here investigated HPA axis hormone profiles before and after two different acute stressors in male and female Sprague-Dawley rats. Taken together, it appears that the effect of sex on HPA axis hormone activation is stressor-specific. These data not only conflicted with our predictions, but also conflict with previous literature demonstrating that females had significantly higher concentration of CORT following just 10 minutes of loud noise (Seale et al, 2004a). It is possible that a sex difference in HPA axis activation after noise stress exists at a time point earlier than 30 min, since this is the earliest time point utilized in these studies. However, for both stressors, we consistently observed that females have higher baseline concentrations of the stress hormones ACTH and CORT, effects that have been cited for approximately 50 years (Kitay 1961; Critchlow et al, 1963). Therefore, it is possible that the previously reported sex difference following such a short duration of noise stress (Seale et al, 2004a) simply reflects initial basal sex differences in HPA axis hormones, and not stress-induced sex differences in HPA axis hormone increases, per se. Indeed,
comparison of both acute restraint and noise stress in the same male and female rats revealed a robust effect of sex on HPA axis hormone concentrations to restraint stress, but no such effect following noise stress. It is important to point out that in this experiment larger magnitude HPA axis hormone concentrations were expected to be observed following 105 dB compared to 80 dB noise. The lack of increase in these hormones to 105 dB noise compared to 80 dB noise likely reflects some adaptation, or habituation of the stress response, since 105 dB was the second noise stressor exposure in the same animals over the course of several weeks.

In these experiments, careful verification of estrous cycle was performed after each stressor, and in experiments specifically targeting the stage of proestrus, animals were eliminated from analyses if they did not fit this stage (cytologically). This stage of estrus was chosen to maximize the chance of observing the expected sex difference in response to noise, since females in proestrus have been shown to have larger HPA axis responses to stress than other stages of the estrous cycle (Viau & Meaney 1991). However, consistent with our previous observation that stage of estrous cycle does not affect hormone release or central activation of stress-related brain regions to acute restraint stress (Babb et al, 2013), no effect of the estrous cycle was observed in any experiment presented here. Female rats, even when in proestrus, do not appear to have higher HPA axis hormone responses to noise stress than males, and in some cases appear to have significantly smaller magnitude responses than males. Furthermore, removal of circulating sex steroids by ovariectomy actually increased HPA axis hormone responses to loud noise, and replacement with 17β-estradiol after this procedure did not augment, but instead reduced HPA axis hormone responses to loud
noise. Together, these data support our previous finding that circulating steroids in intact females do not have an activational, or augmenting, effect on HPA axis hormone responses to acute stress (Babb et al, 2013).

These studies explored several possibilities of a stressor-specific effect of sex. First, the possibility that female rats have a less sensitive auditory system than male rats was tested with pre-pulse manipulation of the acoustic startle response. If this had been the case, it is conceivable that the lack of sex differences observed following noise stress could be attributed to female rats having higher auditory thresholds, leading to blunted HPA axis responses in this sex compared to male rats. However, not only did both sexes have equal suppression (GAP and PPI trials) and facilitation (PPF trials) of the acoustic startle response, but female rats actually had significantly higher baseline responses to the startle stimulus itself, suggesting that if anything, female rats may be behaviorally more sensitive to auditory stimulation compared to male rats, and not vice versa. Second, we tested the intriguing possibility that females could be having a larger HPA axis hormone response to restraint stress (typically given simultaneously in groups of animals in close proximity to each other) compared to noise stress (given in isolation in sound-attenuating chambers) due to the social cues of the stressor that are present or not. The hypothesis tested in this experiment was that the social context during restraint stress (sight, smell, and sound of other rats being stressed simultaneously) was in effect an additional stressor to the rats, and that these cues might have a greater impact in female compared to male rats. However, in contradiction to this hypothesis, the effect of sex did not disappear when male and female rats were given restraint in isolation. Female rats still displayed higher HPA axis hormones to restraint compared
to male rats, regardless of whether the stress occurred in isolation or in close proximity to other rats. Furthermore, if anything, restraint given in isolation tended to increase HPA axis responses rather than decrease the activation of these hormones. Female rats reportedly have different pain sensitivity than male rats (Aloisi et al, 1994; Gamaro et al, 1998; Hurley et al, 2008), therefore we also tested the idea that female rats were displaying higher HPA axis responses to restraint stress due to the pain of blood sampling that typically occurred just prior to the presentation of this stressor. Female rats still displayed higher HPA axis responses to restraint without blood sampling prior to this stressor, suggesting that sex differences in response to restraint are not caused by differential pain processing in male and female rats.

It is possible that restraint is perceived as more stressful than noise stress, and thus brings out sex differences in HPA axis activation that noise stress does not. Experiencing restraint involves more sensory systems than noise stress, which is assumed to be almost exclusively auditory. Restraint can involve not only the auditory system, but also activates many forebrain regions responsive to somatosensory input, including cortical regions, hippocampus, and prefrontal cortex (Chowdhury et al, 2000). We have demonstrated previously that female rats exhibit higher restraint stress-induced activation of the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BST), and the PVN, despite similar activation of brain regions associated with somatosensory processing (Babb et al, 2013). In the present studies we demonstrate that noise can also significantly activate regions involved in stress responses, including the PVN, MPOA, and BST, as well as others that we have previously reported to be activated by noise stress in male rats (Burow et al, 2005).
However, despite significant activation of these stress-responsive brain regions, no significant effect of sex was observed in any of these regions, or in regions involved in auditory processing. Therefore it is unlikely that noise stress does not induce sex differences in HPA axis responses simply because it is less stressful, since it clearly produces a stress response in both sexes, both peripherally in HPA axis hormone concentrations, and centrally in relevant brain regions.

In conclusion, the experiments presented here suggest that for some stressors, such as restraint, sex differences are robust and large in magnitude, where female rats demonstrate consistently higher magnitude of HPA axis responses to acute presentations of this stressor than male rats. However, results obtained herein with loud noise stress illustrate that sex differences are not always present, or can even exist in the opposite direction, where male rats exhibit significantly higher HPA axis responses to acute stress exposures compared to female rats. Whereas the factors responsible for differential effects of stressor modality have not been ascertained, it appears that auditory threshold, social cue, and nociceptive factors are not the primary stimulus dimensions responsible for stressor-specific differences in stress-induced HPA axis activation.
CHAPTER FOUR:

THE EFFECT OF SEX ON HABITUATION TO REPEATED STRESS IN RATS

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Abstract

Sex differences in repeated stress-induced hypothalamic-pituitary-adrenal (HPA) axis habituation could be important for understanding sex-biased rates of certain psychiatric illnesses such as depression. In these studies, male and female rats were exposed to 30 min of either audiogenic or restraint stress daily for ten days in order to determine whether sex regulates the rate at which HPA axis hormone releases is reduced in response to repeated homotypic stressor presentation. In response to the initial exposure, both stressors robustly increased plasma concentrations of both adrenocorticotropic releasing hormone (ACTH) and corticosterone (CORT). Acutely, females displayed higher hormone concentrations after restraint stress, whereas males exhibited higher hormone concentrations following loud noise stress. HPA axis hormone responses to both stressors decreased incrementally over successive days of exposure to the respective stressors. Despite the differential effect of sex acutely following each stressor, the extent to which HPA axis hormone responses in male and female rats was attenuated did not differ for either stressor modality. Furthermore, responses to a novel environment on the eleventh day did not reveal an effect of sex on HPA axis responses in animals exposed to prior stress of either modality. These experiments show that despite differential effects of sex on HPA axis responses to the first presentation of a particular stressor, male and female rats exhibit a similar extent of HPA axis habituation, and repeated homotypic stress does not produce differential effects of sex on subsequent exposure to a novel environment on HPA axis hormone release.
Introduction

Habituation of hypothalamic-pituitary-adrenal (HPA) axis responses is typically defined as a reduction in stress-induced circulating plasma hormone concentration after repeated exposure to the same (homotypic) stress stimulus (Marti & Armario, 1998; Grissom & Bhatnagar, 2009). This is an adaptive and/or protective process for an organism, since exaggerated or prolonged stress responses can be detrimental to one’s somatic and mental health (Chrousos & Gold 1998; Van tallie 2002; Reagan et al, 2008). Indeed, repeated and/or sustained stress responses are associated with the etiology of somatic and psychiatric illnesses in humans, such as major depression (Chrousos & Gold 1998; Ehlert et al, 2001; Claes 2004; McEwen 2004). Several stress-influenced psychiatric illnesses are approximately twice as prevalent in women as in men, including depression (Linzer et al, 1996; Kessler et al, 2005; Van de Velde et al, 2010) and several anxiety disorders (Stein et al, 2000; Holbrook et al, 2002; Tolin & Foa, 2006; Bekker & van Mens-Verhulst, 2007; Olff et al, 2007; Christiansen & Elklit, 2008; Vesga-Lopez et al, 2008; Ditlevsen & Elklit, 2010). Furthermore, HPA axis function is often dysregulated in patients with these psychiatric illnesses (Ehlert et al, 2001; Jones & Moller, 2011; Lloyd & Nemeroff, 2011). HPA axis functions are also known to be modulated by sex steroid hormones (Altemus et al, 1997, Kirschbaum et al, 1999; Altemus et al, 2001). Therefore, many investigators have proposed that sex may be an important susceptibility factor in the differential rate of development of stress-related diseases, possibly due to greater effects of chronic or prolonged stress on dysregulation of the HPA axis in women.
Both loud noise and restraint stress presented acutely can robustly increase the two main hormone outputs of the HPA axis in rodents: adrenocorticotropic releasing hormone (ACTH) and corticosterone (CORT) concentrations (see Chapters 2 and 3), and have long been used to study repeated stress-induced HPA axis habituation (Armario et al, 1984; Natelson et al, 1988; Marti & Armario 1997; 1998; Pace et al, 2001; Sasse et al, 2008; Nyhuis et al, 2010a; 2010b; Weinberg et al 2010). Furthermore, we have used repeated presentations of loud noise stress extensively to investigate the neural circuitry involved in habituation of HPA axis responses in male rats (Campeau et al, 1997; 2002; Masini et al, 2008; Day et al, 2009; Masini et al, 2012). Acutely, female rats typically display markedly higher stress-induced HPA axis hormones following a variety of stressors compared to male rats (Le Mevel et al, 1978; Livezey et al, 1985; Heinsbroek et al, 1991; Aloisi et al, 1994; Handa et al, 1994; Ogilvie & Rivier, 1997; Aloisi et al, 1998; Weinstock et al, 1998; Drossopoulou et al, 2004; Seale et al, 2004a; 2004b; Viau et al, 2005; Larkin et al, 2010; Babb et al, 2013). However, we have demonstrated that the effect of sex on acute stress-induced HPA axis hormones is not always present, and appears to be stressor-specific. Namely, while female rats have a higher HPA axis response to restraint stress, this is not the case for acute noise stress exposure (see Chapter 3). These prior studies demonstrating the differential role of sex on acute stress-induced HPA axis hormones provides a unique opportunity to evaluate the impact of sex on HPA axis hormone habituation, to investigate whether these acute effects also translate into a differential effect of sex following repeated homotypic stress exposures. Because different strains of rats that show different acute responses to stress also display differential habituation
of HPA axis responsiveness over a period of 10 days to that same stressor (Dhabhar, et al, 1997), we hypothesized that female rats, only when exhibiting significantly higher acute HPA axis responses, would also display impaired HPA axis habituation to repeated exposures to that same (homotypic) stressor, as indexed by more prolonged HPA axis increases over successive days compared to male rats.

Several studies have suggested that female rats display different responses to repeated stress than males, including HPA axis activity (Galea et al, 1997; Bowman et al, 2003; Bhatnagar et al 2005). However, some of these studies suggest that female rats do not express HPA axis habituation to repeated stress to the same extent as males (Galea et al, 1997; Bhatnagar et al, 2005), although there have been conflicting reports (Bowman et al, 2001). All of these previous reports, however, utilized repeated restraint stress, where sex differences in acute HPA axis responses are observed. To our knowledge no studies have compared adaptation of HPA axis responses to repeated loud noise stress in male and female rats. Therefore the experiments presented here compare HPA axis responses to repeated presentations of either loud noise (100 dB) or restraint stress for 30 min daily over 10 days in male and female rats. Other male and female rats, which received the same handling/blood sampling procedures but no loud noise or restraint stress exposure, served as controls in both experiments. In addition, studies in male rats have suggested that facilitated or sensitized HPA axis responses to a heterotypic (novel) stressor can be observed following repeated homotypic stress exposures (Marti et al, 1994; Bhatnagar et al, 1998; Marti & Armario, 1998; Andres et al, 1999; Bhatnagar & Vining 2003). Therefore, 24 hours after the last homotypic stressor presentation, the aforementioned groups of
animals were all exposed to a novel environment to test whether female rats would exhibit more exaggerated HPA axis responses to this exposure compared to male rats, and to no stress controls of both sexes. Completely naïve male and female rats that remained untouched other than sporadic body weight measurements throughout the experiment served as further controls for this novel environment exposure given on Day 11. Despite previous reports suggesting that female rats do not display HPA axis habituation (Galea et al, 1997; Bhatnagar et al, 2005), the results presented here suggest that regardless of initial hormone responses, male and female rats both exhibit rapid HPA axis habituation, and that sex does not affect the extent of this adaptation.

**Materials and Methods**

*Subjects*

Young adult (2-5 m.o.) female and male Sprague-Dawley rats weighing 200-225g at the time of arrival were used for all experiments, and were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animals were allowed to acclimatize to the animal facility for one week prior to any experimental manipulation. All animals were housed in clear polycarbonate cages either in same-sex pairs or groups of four with standard wood shavings bedding and *ad libitum* access to food (standard rat chow) and water. All colony rooms were maintained on a controlled 12:12 light:dark cycle (lights on at 7am) under constant temperature and humidity. To coincide with the nadir of the diurnal rhythms of both ACTH and CORT, all experimental procedures were performed between 9:00 am and 12:00 pm. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and
conformed to the National Research Council’s (2011) *Guide for the Care and Use of Laboratory Animals, 8th Edition.*

**Procedure**

In separate experiments, male and female rats were exposed to 10 days of daily 30 min exposure to either loud noise (100 dB) or restraint stress. In both experiments separately, other male and female rats were exposed to handling and blood sampling procedures, but did not receive stress (No Stress Controls). Blood sampling via a lateral tail vein was performed in all of these animals on days 1, 3, 6, and 10. On the 11th day, stress and no stress control animals were exposed to a novel environment for 30 min in the colony room. All animals were sacrificed immediately after the 30 min novel environment exposure, trunk blood was collected for hormone concentration determination, and thymus and adrenal glands were extracted. In both experiments, a third group of male and female rats remained untouched in the colony room over the entire 10-day stress procedure except for body weight measurements (Naïve animals). Naïve male and female rats were also sacrificed on day 11 and blood and tissues were also collected in these animals, but these animals did not receive novel environment exposure like the Stressed and No Stress control male and female rats from both experiments.

**Estrous Cycle Verification**

Prior to any stress presentation, all female rats were monitored daily for estrous cyclicity via vaginal lavage samples examined with light microscopy. Only females displaying at least two full normal 4-5 day estrous cycles were used in each study.
Males in all experiments were handled daily to control for this procedure. Cyclicity of the estrous cycle was not affected by daily presentations of either repeated loud noise or restraint stress, compared to no stress control females (data not shown).

**Noise Stress**

The sound-attenuating acoustic chambers consisted of ventilated double wooden (plywood board) chambers, with the outer chamber lined internally with 2.54 cm insulation (Celotex, Suffolk, England). The internal dimensions of the inner box were 60 cm (w) x 38 cm (d) x 38 cm (h), within which a rat home cage could be placed. The middle of the ceiling of each inner chamber was fitted with a single 6 x 9 inch Optimus speaker (Radio Shack, #12-1769-120 W RMS). Light within the inner chamber was provided by a florescent bulb (15W) covered with red tissue paper in the upper left corner. Two fans (upper left and lower right corners) allowed fresh air to flow into the inner chamber while also removing stale air. Noise was produced by a General Radio (#1381, Concord, MA) solid-state random noise generator with the bandwidth set at 2 Hz-50 kHz. The output of the noise generator was amplified (Pyramid Studio Pro #PA-600x, Brooklyn, NY) and delivered to each speaker. The speaker characteristics allowed sound delivery between 20 and 27,000 Hz, rolling off quickly (20 dB/octave) at both ends. Noise intensity was measured directly under the speaker on the bottom of the inner chamber with a Sound Level Meter (A scale, #33-2050, Radio Shack). The ambient noise level inside the chamber was approximately 60 dB (background noise level), which was approximately 5 dB above the ambient noise level in the colony rooms due to the noise from the air circulating fans within each chamber.
Restraint Stress

Restraint was conducted in clear ventilated, cylindrical Plexiglas tubes of adjustable length. The size of the tube (23.5 cm in length and 7 cm in diameter for the males; 15.5 cm long and 6.3 cm diameter for the females) restricted movement in all directions with the rat’s tail protruding from the cylinder, but did not interfere with breathing.

Novel Environment Exposure

Novel environment exposure consisted of placing rats, individually, into a white circular 5 gallon household plastic bucket (base diameter 25 cm, height 36 cm). This method has previously been used as a low-intensity stressor in male rats (Campeau et al, 2010).

Blood collection

For tail blood collection, animals were gently held immobile using a clean towel, and a small incision was made in a lateral tail vein with the corner of a razor blade. Blood was collected using heparinized hematocrit capillaries deposited into ice-chilled 0.5mL microcentrifuge tubes (for CORT assays), and using uncoated hematocrit capillaries deposited into similar tubes containing EDTA (20 mg/ml) for ACTH assays. This procedure lasted less than 4 min to prevent detection of elevated hormone levels due to the blood collection procedure itself. These blood samples were centrifuged at 14,000 rpm at room temperature for 1 min. After the final stressor exposure, rats were decapitated and trunk blood was collected into large EDTA-coated tubes (Vacutainers). These tubes were then centrifuged at 2,000 rpm for 10 min at 4°C. For all samples,
Plasma was then pipetted into 0.5ml Eppendorf microcentrifuge tubes and stored at -80°C until assayed for hormone concentrations.

**ACTH IRMA**

Plasma ACTH concentrations were assayed using a commercially available immunoradiometric assay kit (Cat # 27130; DiaSorin, Stillwater, MN, USA); which has a sensitivity of 1.5 pg/ml, and inter- and intra-assay coefficients of ~4.6% and ~ 4.18%, respectively. This IRMA kit recognizes 100% of whole molecule ACTH (detects all 39 amino acids), and as such is very specific, with undetectable cross-reactivity for α- and β-MSH, as well as β-Endorphin. The assay was performed according to the kit directions. Briefly, 200 µl of plasma or plasma diluted with zero standard (provided with the kit) was incubated overnight with a $^{125}$I-labelled monoclonal antibody specific for ACTH amino acids 1-17, a goat polyclonal antibody specific for ACTH amino acids 26-39, and a polystyrene bead coated with a mouse anti-goat IgG. Only ACTH in the sample with all 39 amino acids bound both antibodies to form an antibody complex on the polystyrene bead. The following day, tubes containing the radioactive beads were washed to remove any unbound reagents and the amount of radioactivity still left on each bead was determined with a gamma counter. The concentration of ACTH for each sample was calculated by fitting values for each sample into a standard curve of known ACTH dilutions processed concurrently.

**CORT ELISA**

Plasma CORT concentrations were analyzed using a commercially available kit (Cat. # 901-097; Assay Designs, Ann Arbor, MI, USA). This is a highly specific assay
(100% cross-reactivity with CORT, 28.6% with deoxycorticosterone, and 1.7% with progesterone), with a sensitivity of 26.99 pg/ml, and inter-/intra-assay variabilities of ~7.5% and ~9.7%, respectively. The kit directions were followed with the exception of a modification to use a smaller volume of plasma as follows. The steroid displacement reagent (0.5 µl/ml; provided with the kit) was added to the assay buffer. Ten µl of plasma was then diluted 1:50 with the amended assay buffer. The diluted plasma samples were then processed according to the kit directions. This method was found to result in assayed CORT levels equivalent to the method in the kit directions (data not shown). Briefly, plasma samples and diluted CORT standards were incubated in a 96-well plate coated with anti-sheep antibody raised in donkey, together with CORT with a covalently attached alkaline phosphatase molecule, and a sheep polyclonal antibody for CORT. After a 2 hr incubation period the plate was washed to remove all unbound reagents and a substrate was added. After 1 hr the color reaction was stopped and the intensity of color in each well was analyzed at 405 nm using a microplate reader (Biotek EL808; Winooski, VT, USA). The concentration of CORT for each sample was calculated by fitting unknown values into a standard curve of known CORT dilutions processed concurrently.

Statistical Analysis

All habituation data were analyzed with repeated measures ANOVA, with sex and day as the independent variables. Further post-hoc analyses, when necessary, were performed using independent samples t-tests. Hormone responses to the novel environment were analyzed with a two-way ANOVA with sex and stress condition as
independent variables. SPSS Statistics (version 18 for Windows) was used for all statistical analyses. Significance was set at $p = 0.05$ for all statistical tests.

**Results**

*Body Weights across repeated noise or restraint*

Body weight measurements across both repeated stress experiments are displayed in Figure 4.1. A repeated measures ANOVA on body weight across repeated noise exposures revealed that overall, all animals gained weight over the experiment as demonstrated by a significant main effect of day ($F_{4,72} = 277.57, p < 0.001$; Figure 4.1 A and C). Also, overall males weighed more than females, as demonstrated by a main effect of sex ($F_{1,43} = 631.13, p < 0.001$). A main effect of stress condition was also revealed ($F_{2,43} = 3.97, p < 0.05$), and post-hoc analyses showed that this effect was due to Naïve animals weighing significantly more than No Stress, but not Stressed animals ($p = 0.006$). Stressed animals overall did not differ significantly in weight from either Naïve or No Stress animals ($p$'s $> 0.05$). The same repeated measures ANOVA also revealed significant day x stress condition ($F_{8,172} = 4.77, p < 0.001$) and day x sex ($F_{4,172} = 131.53, p < 0.001$) interactions, but not significant stress condition by sex ($F_{2,43} = 1.33, p = 0.28$) or three-way day x stress condition x sex ($F_{8,172} = 1.49, p = 0.17$) interactions. Thus overall, males gained weight faster than females over the experiment, and that the rate of weight gain over time depended on stress condition. Importantly though, the effect of stress condition on body weight gain across the experiment did not depend on sex, demonstrating that stress did not affect body weight of male and female rats differently.
Mean (± 1 SEM) body weights (g) for female (A and B) and male (C and D) rats exposed to repeated noise (A and C) or restraint (B and D) stress. Repeated stress (Stress) was given daily on Days 1-10. No stress control animals (No Stress) received the same blood sampling procedure as stressed animals, and naïve control animals (Naïve) did not receive blood sampling and were only handled in order to obtain body weights. Male rats overall weighed significantly more than female rats for both stressors (p’s < 0.05).

For the repeated restraint experiment, body weight of naïve animals was only recorded at arrival and on Day 10 (Figure 4.1 B and D). Therefore, two repeated measures ANOVAs were performed- one comparing arrival with day 10 across all 3
stress conditions, and one comparing Stress and No Stress animals across all time points. Comparing Stress and No Stress animals across the experiment, all animals gained weight over time as demonstrated by a significant main effect of day ($F_{4,112} = 544.33, p < 0.001$). Males not only weighed significantly more than females overall as demonstrated by a significant main effect of sex ($F_{1,28} = 336.68, p < 0.001$), but also gained weight significantly faster than females over time, as demonstrated by a significant interaction between day and sex ($F_{4,112} = 182.04, p < 0.001$). Body weight overall did not differ significantly based on stress condition ($F_{1,28} = 1.76, p = 0.19$), nor did it differ over time based on stress condition as demonstrated by a non-significant day by stress condition interaction ($F_{4,112} = 1.5, p = 0.21$), meaning that stress by itself did not affect body weight or body weight gain. There was no significant difference in the effect of stress condition on body weight depending on sex ($F_{1,28} = 0.24, p = 0.63$) meaning that the effect of sex on body weight did not differ based on stress condition. There was also no significant three-way day x stress condition x sex interaction ($F_{4,112} = 1.28, p = 0.28$). Comparing naïve, no stress, and stress animals at arrival and on day 10, there was also no main effect of stress condition ($F_{2,44} = 2.86, p = 0.07$), demonstrating that overall handling (both stress and no stress groups) did not affect body weight, however a significant day by stress condition by sex three-way interaction ($F_{2,44} = 4.62, p = 0.015$) demonstrates that handling had more of an effect on body weight gain over time in male animals compared to female animals.

*HPA axis responses to repeated noise*

Overall, repeated measures ANOVAs revealed a similar pattern of the effects of time (Day 1, 3, 6, and 10), stress condition (Noise or No Stress), and sex (Male and
Figure 4.2

HPA axis hormone concentrations in response to 10 days of repeated noise stress (100 dB for 30 min/day) in male and freely cycling female rats. A) Mean ACTH concentrations (± 1 SEM). B) Mean CORT concentrations (± 1 SEM).
Female) on both ACTH and CORT concentrations, which are displayed in Figure 4.2. Overall, there was a significant effect of Day on both ACTH ($F_{3,81} = 26.38, p < 0.001$) and CORT ($F_{3,81} = 16.68, p < 0.001$) concentrations. In addition noise stress significantly increased both ACTH ($F_{1,27} = 14.88, p = 0.001$) and CORT ($F_{1,27} = 29.22, p < 0.001$) concentrations overall. Furthermore, a significant interaction of day by stress condition existed for both ACTH ($F_{3,81} = 14.21, p < 0.001$) and CORT ($F_{3,81} = 16.74, p < 0.001$) concentrations. Further analysis revealed that noise stress only significantly increased ACTH concentrations on Day 1 compared to controls. Importantly, there was no overall effect of sex on either ACTH ($F_{1,27} = 0.08, p = 0.78$) or CORT ($F_{1,27} = 0.41, p = 0.53$) concentrations, and the effect of sex on HPA axis hormones did not differ across time, as demonstrated by non-significant interactions of day by sex on ACTH ($F_{3,81} = 1.42, p = 0.24$) and CORT ($F_{3,81} = 0.63, p = 0.60$). However, significant sex by stress condition interactions existed for both ACTH ($F_{1,27} = 4.25, p = 0.049$) and CORT ($F_{1,27} = 5.31, p = 0.03$) concentrations. Overall stressed males had higher HPA axis hormone concentrations than stressed females, but control females had higher HPA axis hormone levels than control males. There was not a significant three-way interaction between day, sex, and stress condition on either ACTH or CORT concentrations to repeated noise ($p$'s $> 0.05$).

**HPA axis responses to repeated restraint**

The effects of sex and repeated restraint stress over time are displayed in Figure 4.3. Overall, restraint stress increased HPA axis hormone concentrations in all animals, as demonstrated by a main effect of stress condition on both ACTH ($F_{1,28} = 19.41, p < 0.001$) and CORT ($F_{1,28} = 42.35, p < 0.001$) concentrations. HPA axis hormones overall
Figure 4.3

HPA axis hormone concentrations in response to 10 days of repeated restraint stress (30 min/day) in male and freely cycling female rats. A) Mean ACTH concentrations (± 1 SEM). B) Mean CORT concentrations (± 1 SEM).
were also significantly lowered over successive days of testing, as demonstrated by a significant main effect of day on both ACTH ($F_{3,84} = 31.18, p < 0.001$) and CORT ($F_{3,84} = 27.18, p < 0.001$) concentrations. Sex overall did not affect ACTH concentrations ($F_{1,28} = 3.88, p = 0.06$), although females had significantly higher CORT concentrations compared to males overall ($F_{1,28} = 23.90, p < 0.001$). Importantly though, sex did not affect the rate of HPA axis hormone habituation to repeated restraint stress, as there was no significant interaction of sex and time on either ACTH or CORT concentrations ($p$'s > 0.05). The effect of repeated restraint on HPA axis hormones differed significantly over time, as demonstrated by significant day by stress condition interactions on both ACTH ($F_{3,84} = 19.69, p < 0.001$) and CORT ($F_{3,84} = 29.48, p < 0.001$) concentrations. Unlike repeated noise stress (see Figure 4.2), females had higher HPA axis hormones regardless of stress condition, as demonstrated by non-significant interactions of sex and stress condition on both ACTH and CORT concentrations ($p$'s > 0.05; Figure 4.3). There was not a significant three-way interaction between day, sex, and stress condition on either ACTH or CORT concentrations to repeated restraint ($p$'s > 0.05).

Organ weights across repeated stress

Table 4.1 displays the effect of sex and repeated stress on raw adrenal and thymus weights, although analysis was performed on these weights as a percentage of each rat’s body weight. A two-way ANOVA revealed that females overall had significantly higher percentages of both adrenal ($F_{1,48} = 66.51, p < 0.001$) and thymus ($F_{1,48} = 34.16, p < 0.001$) weight to total body weight following noise stress compared to males. Females also had significantly higher percentages of both adrenal ($F_{1,49} =$
Table 4.1. Effect of sex on adrenal and thymus weight following repeated stress.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>Noise Stress</th>
<th></th>
<th>Restraint Stress</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adrenals</td>
<td>Thymus</td>
<td>Adrenals</td>
<td>Thymus</td>
</tr>
<tr>
<td>Male</td>
<td>Naïve</td>
<td>0.049 ± 0.002</td>
<td>0.55 ± 0.04</td>
<td>0.052 ± 0.002</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>No Stress</td>
<td>0.045 ± 0.002</td>
<td>0.47 ± 0.01</td>
<td>0.046 ± 0.001</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>0.047 ± 0.001</td>
<td>0.51 ± 0.03</td>
<td>0.045 ± 0.002</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Female</td>
<td>Naïve</td>
<td>0.057 ± 0.002(^\wedge)</td>
<td>0.31 ± 0.01</td>
<td>0.064 ± 0.002(^\wedge)</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>No Stress</td>
<td>0.057 ± 0.001(^\wedge)</td>
<td>0.29 ± 0.01</td>
<td>0.059 ± 0.001(^\wedge)</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Stress</td>
<td>0.067 ± 0.002(*\wedge)</td>
<td>0.31 ± 0.02</td>
<td>0.060 ± 0.001(^\wedge)</td>
<td>0.27 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as means ± 1 SEM (in grams). \(^*\) = \(p < 0.05\) compared to naïve and no stress animals for the same sex. \(^\wedge\) = \(p < 0.05\) compared to males.

131.24, \(p < 0.001\) and thymus (\(F_{1,49} = 75.69, p < 0.001\)) weight to total body weight following restraint stress. Because of these robust sex differences, we analyzed the effect of stress condition on organ weights separately for each sex. For females, there was a significant effect of stress condition on adrenal weight following both noise stress (\(F_{2,24} = 8.79, p = 0.002\)) and restraint stress (\(F_{2,24} = 5.55, p = 0.01\)), but no effect of stress on thymus weight following either stressor (\(p's > 0.05\)). Following repeated noise, stressed females had significantly higher adrenal weight compared to both Naïve and No Stress control females (\(p's < 0.05\)). Naïve and No Stress control females did not have different adrenal weight (\(p > 0.05\)). Following repeated restraint, naïve females had significantly higher adrenal weight compared to No Stress control females (\(p = 0.01\)), but did not differ significantly from Stressed females (\(p > 0.05\)). Stressed and No Stress control females did not have significantly different adrenal weights on
Day 11 \( (p > 0.05) \). In males, there was no significant effect of stress on either adrenal or thymus weight following either stressor \( (p's > 0.05) \).

**HPA axis responses to a novel environment following repeated stress**

Figure 4.4 displays the effect of sex and 10 days of prior stress on HPA axis responses to a novel environment exposure given 24 hours after the last stressor exposure (Day 11) to all animals except for naïve controls. Following repeated noise, a two-way ANOVA revealed a main effect of stress condition on both ACTH \( (F_{2,48} = 7.99, p = 0.001; \text{Figure 4.4 A}) \) and CORT \( (F_{2,48} = 10.22, p < 0.001; \text{Figure 4.4 C}) \) concentrations. Post-hoc analyses demonstrated that animals exposed to the novel environment had higher ACTH and CORT concentrations \( (p's < 0.05) \), but importantly, prior stress history did not affect ACTH or CORT concentrations following novel environment exposure \( (p = 0.58) \). Furthermore, there was no significant main effect of sex or sex by stress condition interaction on either ACTH or CORT concentrations following repeated noise stress \( (p's > 0.05) \). However, specific comparison of naïve animals revealed that females had significantly higher ACTH and CORT concentrations than males \( (p's < 0.05) \). Following repeated restraint, a two-way ANOVA revealed a main effect of stress condition on ACTH \( (F_{2,49} = 7.42, p = 0.002; \text{Figure 4.4 B}) \) and CORT \( (F_{2,49} = 9.77, p < 0.001; \text{Figure 4.4 D}) \) concentrations. Post-hoc analyses showed that animals exposed to the novel environment had higher ACTH and CORT concentrations \( (p's < 0.05) \), but again, prior stress history did not affect ACTH or CORT concentrations following novel environment exposure \( (p = 0.36) \). In addition, in this experiment females had significantly higher overall concentrations of both ACTH \( (F_{1,50} = 5.52, p = 0.02) \) and CORT \( (F_{1,50} = 15.81, p < 0.001) \) concentrations compared to males.
Figure 4.4

Mean ACTH (A and B) and CORT (C and D) concentrations (± 1 SEM) in response to a novel environment for 30 min presented 24 hours after the last repeated noise (A and C) or restraint (B and D) exposure in male and female rats. Naïve control animals (Naïve) were sacrificed without being exposed to the novel environment. Novel environment exposure (NE) significantly increased ACTH and CORT concentrations in all animals in both experiments (p’s < 0.05). Prior Stress (Noise or Restraint) did not affect HPA axis responses to NE (p’s > 0.05). * = p < 0.05 compared to male rats in the same stress condition.
However, there was no significant interaction of sex by stress condition on either ACTH or CORT concentrations ($p$'s > 0.05), demonstrating that the effect of sex on HPA axis hormones was equivalent regardless of novel environment exposure. Furthermore, specific comparison of male and female rats exposed to novel environment using t-tests revealed no effect of sex on ACTH concentrations with or without prior stress exposure ($p$'s > 0.05). However, naïve females had significantly higher concentrations of ACTH ($t_{16} = 2.47, p = 0.02$) compared to naïve males. Specific comparison of CORT concentrations revealed that females had significantly higher CORT concentrations compared to males across all three experimental conditions ($p$'s < 0.05).

**Discussion**

The data presented here investigated the effect of sex on the extent of HPA axis hormone habituation to repeated stress. We have previously demonstrated a robust effect of sex on HPA axis hormone concentrations in response to acute restraint stress (Babb et al, 2013), and that this robust effect of sex is not present in response to acute noise stress (Chapter 3). Here we have confirmed our previous results, demonstrating that the female HPA axis appears to be more responsive to restraint, but not noise stress acutely compared to the male HPA axis. The results from this study suggest that males might actually be more sensitive to acute noise stress-induced responses of the HPA axis compared to females. Despite the observation that central indices of the HPA axis are also more highly activated in the female compared to male brain after restraint stress (Babb et al, 2013), it appears that sex does not affect stress-induced habituation of either ACTH or CORT concentrations following either noise or restraint stress, regardless of the effect of sex following an initial (acute) presentation of a stressor. This
may suggest that the neurocircuitry in male and female brain involved in HPA axis habituation is similar. Future research is needed to test this hypothesis.

Here we tested whether female rats, given the observation of higher magnitude stress-induced HPA axis hormones to at least some acute stressors, would also show impaired or delayed habituation of HPA axis hormone responses to repeated homotypic stressors over successive days. Evidence that this might have been the case comes from the observation that rat strains that display differential acute stress-induced HPA axis hormones also display differential habituation of HPA axis hormones in responses to repeated presentations of a stressor (Dhabhar et al, 1997). However, regardless of whether female rats displayed higher (restraint) or lower (noise) acute stress-induced HPA axis responses, these acute effects did not translate into either impaired or improved habituation of HPA axis responses to repeated stress in female compared to male rats. By and large, the rate and final level of ACTH and CORT habituation observed in males and females was comparable. These results also show that female rats do not display a greater facilitation of HPA axis responses to a subsequent novel stressor following repeated homotypic stress compared to male rats.

Previous studies performed in male rats have demonstrated either normal or facilitated HPA axis responses to a novel stressor after repeated exposures to a homotypic stressor (Bhatnagar et al, 1995; Akana et al 1997; Bhatnagar & Dallman 1998; Marti & Armario 1998; Bhatnagar & Vining 2003; Grissom et al, 2008). We have previously used the same novel environment used in this study to investigate the effect of voluntary exercise on HPA axis responses to low intensity stressors (Campeau et al, 2010; Masini et al, 2011), and have also observed sensitized HPA axis responses in
male rats to the same novel environment following three days of 10 s-long, 1mA footshocks each day (Day HEW and Campeau S, unpublished observations). No sensitized HPA axis responses to novel environment presentation were observed in animals previously exposed to either noise or restraint stress compared to control animals that received the same handling and blood sampling procedure. However, all animals that were exposed to the novel environment did display significantly higher HPA axis responses than naïve control animals, suggesting that the novel environment by itself did induce HPA axis hormone activation. Most importantly though, is that sex did not interact with prior stress exposure to produce differential HPA axis responses to a novel environment. That is, female rats were not more negatively affected by prior stress as compared to male rats. Female rats displayed higher CORT, although similar ACTH, concentrations to novel environment exposure following restraint stress compared to male rats, and this may reflect sex differences in basal HPA axis hormone concentrations rather than stress-induced levels, since the same effect of sex on CORT concentrations following noise stress was not observed. It is unlikely that this effect was observed due to restraint being a more intense stressor, since acute HPA axis hormone concentrations were nominally higher in response to noise compared to restraint stress.

It is possible that facilitated HPA axis responses to novel environment exposure were not observed due to the intensity of the homotypic stressors used. Although both male and female naïve animals displayed faster body weight gain in general for both experiments, handling alone appeared to have affected body weight gain to the same degree as repeated stressor exposure, since No Stress and Stress male and female groups displayed similar body weight gain in general. In addition, adrenal hypertrophy
and thymic involution, two physiological hallmarks of severe stress experience, were not observed in animals exposed to repeated noise or restraint stress, suggesting that neither of these stressors was very severe. Female rats exposed to repeated noise stress did exhibit significantly higher percentage of adrenal to body weight, although the same effect was not observed in male rats exposed to repeated noise even though this group displayed significantly higher HPA axis hormone responses to this stressor acutely, and female rats exposed to repeated restraint stress exhibited reduced percentage of adrenal to body weight compared to naïve female rats. Taken together, this suggests that although small effects of repeated stress existed, handling of animals resulted in body, adrenal, and thymus weights that were quantitatively similar, despite markedly different HPA axis responses, at least for the first few days of stressor exposure. Regardless, the lack of robust sex differences on HPA axis responses to novel environment exposure likely was not due to the intensity of the repeated homotypic stressor used, since female HPA axis responses, to acute restraint stress at the least, are robustly higher than the male counterpart (Babb et al 2013 and the current study).

We conclude that despite differential effects of sex on acute HPA axis hormone responses to stress, male and female rats have the same rate of habituation of these hormones following repeated presentations of the same stressor. Furthermore, previously stressed rats do not display sensitized HPA axis hormone concentrations to a novel environment compared to control animals, although an effect of sex may exist on this measure. These data suggest that sex differences in the prevalence of certain
stress-related psychiatric illnesses are likely not due to differential effects of sex on HPA axis adaptation to repeated homotypic stress.
CHAPTER FIVE:

GENERAL DISCUSSION
Summary of results

The data presented here demonstrate that when sex differences are observed in HPA axis hormone responses to acute stress, as in the case of acute restraint, these effects translate into significant differences not only in the primary effector of the HPA axis, the PVN, but also in other stress-related brain regions known to influence HPA axis function. Specifically, Chapter 2 reveals significantly higher activation of neurons in the female PVN following restraint, specifically in CRH-producing neurons within this region. In addition, higher HPA axis hormone release following acute restraint is also accompanied by significantly higher activation of both the BSTav and the MPOA in the female brain, although the majority of this activation comes from non-CRH-producing neurons, as demonstrated in Chapter 2. The phenotype of the neurons that are more highly activated in the female BSTav and MPOA are of great interest, and the primary neuropeptide(s) these cells produce, whether these cells are projecting directly to the PVN, to other stress-responsive brain regions, or to brain regions currently not associated with stress responsiveness in the male brain, are yet to be determined. As discussed in the Introduction, the BST and MPOA are regions that generally express high levels of gonadal hormone receptors, which could be critical to help explain the observed sex differences in response to some stressors, such as restraint. Although no effect of circulating hormones in the female were observed, it is still possible that differential gonadal hormone receptor expression in activated neurons of the BSTav and MPOA play some role in differential HPA axis activation under some conditions, as proposed by Williamson et al, 2010, and this could be a target of future studies. Importantly, the sex difference observed in stress-related brain regions is not also
accompanied by higher activation of sensory regions in the female brain that are likely to be responsive to restraint, such as the barrel field area of primary somatosensory cortex and thalamic somatosensory regions, which receive input from the whisker area and other areas of the body of the rat. Therefore differential activation of primary sensory regions is not likely to be the mechanism of sex differences in HPA axis hormone responses observed following restraint.

Conversely, Chapter 3 demonstrates that when sex differences in HPA axis hormone responses are NOT observed (at least consistently and in the dogmatic direction), as is observed following acute noise, the female brain also responds similarly, displaying comparable activation of the PVN, BSTav, MPOA, and many other stress-related regions, and that sensory regions involved in auditory processing also do not show an effect of sex in the magnitude of their activation. As described in the Introduction, due to the large volume of studies that have reported the same finding, the dogmatic idea from the literature is that female rodents always display higher stress-induced HPA axis hormones no matter what stressor is utilized, and that this effect translates into the central processing of the HPA axis (Le Mevel et al, 1978; 1979; Haleem et al, 1988; Heinsbroek et al, 1991; Aloisi et al, 1994; 1998; Weinstock et al, 1998; Rivier 1999; Panagiotaropoulos et al, 2004; Seale et al, 2004a; 2004b; Viau et al, 2005). The HPA axis hormone and brain activation results presented here contradict this dogmatic idea. Furthermore, differential processing of auditory stimuli cannot explain the lack of the dogmatic effect, since sensitivity of the female acoustic startle reflex to modulation by several types of pre-pulses, as well as the aforementioned activation of auditory processing regions following acute noise, are comparable. In
addition, the threshold of noise intensity needed to activate the HPA axis, or the extent of HPA axis hormone responses after the termination of noise, do not appear to be affected by sex. Two possibilities were tested as to why these two stressor modalities produce differential effects of sex: that restraint given in a group, or that basal tailnicks given prior to restraint, caused higher activation of the HPA axis in female compared to male rats. However, neither of these factors appears to be the explanation. More research is needed to understand why and how different stressor modalities can produce differential effects of sex on stress-induced HPA axis function. It should be noted that taken together, these studies (presented in Chapters 2 and 3) are the first to rule out the possibility that differences in primary sensory perception are likely providing the basis for differences in stress responsiveness between sexes.

Chapter 4 utilized the differential effect of sex observed on HPA axis responses to acute stressor presentation in order to investigate whether sex also affects the extent to which HPA axis responses will adapt, or habituate, to repeated presentations of the same (homotypic) stressor, and whether that adaptation would lead to facilitation of HPA axis responses to a novel (heterotypic) stressor, differentially in male and female rats. What is clear is that sex does not affect the extent to which HPA axis responses habituate, or HPA axis responses to a mild heterotypic stressor following this adaptation. Importantly, this lack of effect of sex on HPA axis response habituation was observed following repeated presentations of either restraint or noise, acute presentations of which were shown to elicit differential effects of sex. As discussed in the Introduction, one potential factor mediating differential susceptibility of men and women to various stress-related physical and mental illnesses could be the ability to
adapt to repeated exposures to stress. However, the results of Chapter 4 suggest that the reduction of HPA axis responses to repeated homotypic stress exposures is similar in rats of both sexes, and that this pattern is consistent across different stress situations. It is possible still possible, however, that responses to repeated heterotypic stress situations may lead to differential responses observed between the sexes.

Unlike many previously published reports demonstrating stimulatory effects of female sex steroids, no such effects of exogenous estradiol, or even endogenous changes across the estrous cycle were observed in any of the experiments presented here. Many years ago, it was suggested that small amounts of estradiol are stimulatory to the HPA axis, but that large amounts of estradiol show the opposite effect, and are inhibitory to the HPA axis (Kitay, 1963a). The data herein do not support this hypothesis. Perhaps estradiol truly does not augment the function of the HPA axis, either at rest or following stress. However, an alternative hypothesis that could explain the lack of observed effects of either endogenous or exogenous estradiol on HPA axis is that female rats, and male rats for that matter, used in these experiments are experiencing high enough levels of estrogens that either no effect of this hormone on HPA axis responses is observed, or that the HPA axis is even being inhibited by the levels of this hormone these rats are experiencing. One proposed explanation for how this could be occurring is that all animals in these studies are being exposed to phytoestrogens through their diet. Soy is indeed the first ingredient in the rat chow used in the colony in which the subjects in this dissertation were maintained. Soy contains several isoflavones, the main two being genistein and daidzein, that are known endocrine disruptors, and these chemicals have been proven to affect several behaviors
in rodents, including anxiety-like behavior (Lephart et al, 2004). It is therefore possible that due to the dietary estrogenic chemicals these rats were exposed to, any normal effects of estrogens on female HPA axis function were muted, or even reversed. Any potential effects in the male are more difficult to ascertain since no measurement of testosterone concentrations was performed in these experiments, however it is possible that dietary soy was also affecting the male HPA axis. However, in the case of acute restraint stress at least, dietary soy did not disrupt the expression of sex differences in HPA axis hormone release and stress neurocircuitry activation. Because no direct measurement of the potential effects, if any, dietary soy isoflavones were exerting on HPA axis function in these experiments, this idea is purely speculative. Importantly though, a simple explanation of the effect of sex on HPA axis, and perhaps additional stress-induced responses based solely on differential expression of gonadal hormone receptors that some have suggested (Bingham et al, 2006; Williamson et al, 2010) is made more complex given the inconsistencies in these effects observed across different stress modalities (see Chapter 3).

**Further implications for human mental health and future research**

Overall, these results have several implications for the effect of sex on stress responses in humans. The fact that the BST was one of the brain regions found to be more highly activated in female rats following acute restraint in Chapter 2 might not only be important in the regulation of HPA axis activity, but could have broader implications in the stress-induced regulation of fear and anxiety, due to the reviewed importance and involvement of this region in these specific affective states. Indeed, women show more sustained anxiety, but not phasic fear responses, than men, an effect that is mimicked
by the rodent literature, and that the BST is implicated in mediating (Grillon et al, 2008). In addition, a recent study showed that in both human women and female rats, fear extinction is impaired by reducing estrogen concentrations with hormonal contraceptives (given to both species), an effect that can be reversed by administration of estradiol, also in both species (Graham & Milad, 2013). Other studies have supported the idea that estrogens affect the expression of fear and anxiety (Toufexis et al 2005; 2006; 2007) and perhaps the many studies that show a facilitating effect of estrogen on HPA activity were actually measuring the augmenting effect of estrogen on these affective states. The fact that fear and anxiety states are paralleled so closely in humans and rodents underscores the importance of investigation into stress-related neurocircuitry that may mediate these emotions, such as the BST. The results from Chapter 2 support the idea that higher activation of the female BST, and possibly also of the MPOA, could be important for stress responsiveness irrespective of the function of the HPA axis.

The apparent conservation of neurocircuitry involved in fear and anxiety from rodents to humans just described is in stark contrast to the oppositional effects that sex appears to have on HPA axis function in these two species. Namely, the dogmatic thought from previously published literature in rodents is that higher HPA axis responses are observed in females (Le Mevel et al, 1978; 1979; Haleem et al, 1988; Heinsbroek et al, 1991; Aloisi et al, 1994; Aloisi et al, 1998; Weinstock et al, 1998; Rivier 1999; Panagiotaropoulous et al, 2004; Seale et al, 2004a; 2004b; Viau et al, 2005), and the dogmatic thought in the human literature is that males exhibit higher stress-induced HPA axis responses than women (Kirschbaum et al, 1992; Kudielka et al, 1998; Kirschbaum et al, 1999; Uhart et al, 2006; Shalev et al 2009; Paris et al 2010).
As mentioned in Chapter 1, the TSST is practically the only stressor utilized to study the effect of sex on HPA axis function in humans (Kirschbaum et al, 1993), but as the data from Chapter 3 illustrate, the effect of sex on HPA axis activity cannot be generalized as representative of stress-induced HPA axis responses to all stressors. In fact, Chapters 3 and 4 demonstrate that sex can have practically opposite effects on HPA axis responses to acute stress of different modalities. This begs the question as to whether another modality of laboratory stress may cause the opposite effect of sex on acute HPA axis responses in humans to be observed. Were this to be the case, it would lend an explanation to the seemingly contradictory effects of sex on stress-induced HPA axis function in these two species. Ironically, observations of salivary cortisol responses to an unexpected intermittent construction noise suggested that women exposed to the noise stress had higher cortisol responses compared to males exposed to the same stressor, although in those who were not exposed to the noise stressor, men displayed the typical higher basal cortisol levels compared to women (Paris et al, 2010). Although the HPA axis is just one of a myriad of systems responsive to stress on which sex could be exerting an influence, the clinical literature warrants attention to this system in particular due to its apparent involvement in mood and anxiety disorders. These results therefore illustrate the need for more studies utilizing different modalities of psychological stress in humans.
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