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Sex Differences in Stress-Responsive Neural Substrates and the Development of Mood Disorder-Like Behavior Following a Rodent Model of Early-Life Adversity

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SEX DIFFERENCES IN STRESS-RESPONSIVE NEURAL SUBSTRATES AND THE DEVELOPMENT OF MOOD DISORDER-LIKE BEHAVIOR FOLLOWING A RODENT MODEL OF EARLY-LIFE ADVERSITY

by

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Under the Direction of Nancy G. Forger PhD

ABSTRACT

Stress-related mood disorders, such as anxiety and depression, are the most common psychiatric conditions, occurring with a lifetime risk of 15-20%. Women are twice as likely to develop anxiety and depression than men, and this sex difference emerges during puberty. Exposure to abuse or maltreatment during early life increases mood disorder susceptibility, suggesting that females may be especially sensitive to long-lasting, negative effects of early-life stress. While the female-bias in mood disorders is one of the most robust sex differences in psychiatry, the origin of this difference remains unknown. Sexually dimorphic processing of stressors by the adolescent brain, or the sex-specific expression of stress-related neural

substrates, may be mechanisms by which stress-related mood disorders are more prominent in females. We developed a novel animal model of early-life adversity, Juvenile Social Subjugation (JSS), to test the effect of chronic adolescent social stress on mood disorder-like pathology in adulthood. This dissertation addressed the following research questions: (1) Does chronic JSS induce sex-specific anxiety and depression-like behaviors and HPA axis dysfunction in adulthood? (2) Is JSS differentially processed by the male and female adolescent brain? (3) Is the corticotropin-releasing factor receptor (CRF) system sex-specifically expressed across development? Together our data point to regional sex differences in neuronal activation and CRF receptor expression in the brain as potential mechanisms by which stressors such as JSS induce sex-specific mood disorder-like behavior in adulthood.

INDEX WORDS: Stress, Sex differences, Mood disorder, Anxiety, Depression, Animal model, Puberty, Hypothalamic-pituitary-adrenal axis, Corticotropin-releasing factor, Amygdala

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DEDICATION

This dissertation is dedicated to my mother, Susie Lynn Greer, who always believed in me, and frequently reminded me of what is possible - anything.

"Logic will get you from point A to point B. Imagination will take you everywhere." **Albert Einstein**

"Every great and deep difficulty bears within itself the solution. It forces us to change our thinking in order to find it." **Niels Bohr**

"Sometimes life hits you in the head with a brick. Don't lose faith." **Steve Jobs**

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LIST OF ABBREVIATIONS

AcbC, Nucleus accumbens, core

AcbS, Nucleus accumbens, shell

ACC, Anterior cingulate cortex

BC, Benign social control experience

BLA, Basolateral amygdala

BLAa, Basolateral amygdala, anterior subdivision

BNSTad, Bed nucleus of the stria terminalis, anterodorsal subdivision

BNSTal, Bed nucleus of the stria terminalis, anterolateral subdivision

BNSTif, Bed nucleus of the stria terminalis, interfascicular subdivision

BNSTpr, Bed nucleus of the stria terminalis, principal subdivision

CA3, Cornu ammonis, layer 3

CeA, Central amygdala

CORT, Corticosterone (rodents) and cortisol (humans)

CRF, Corticotrophin releasing factor receptor

CRF1, Corticotrophin releasing factor receptor, type 1

CRF2, Corticotrophin releasing factor receptor, type 2

CRFR, Corticotrophin releasing factor receptor (subtype unspecified)

DG, Dentate gyrus

ELS, Early-life stress

EPM, Elevated plus maze

FST, Forced swim test

HPA, Hypothalamic-pituitary-adrenal

JSS, Juvenile social subjugation

LH, Luteinizing hormone

LSd, Lateral septum, dorsal subdivision

LSi, Lateral septum, intermediate subdivision

LSvl, Lateral septum, ventrolateral subdivision

MeA, Medial amygdala

MePD, Medial amygdala, posterodorsal subdivision

MePV, Medial amygdala, posteroventral subdivision

OT, Olfactory tubercle

PVN, Paraventricular hypothalamus

PIR, Piriform cortex

RA, Resident aggressor

SIT, Social interaction test

T, Testosterone

VMHdm, Ventromedial hypothalamus, dorsomedial subdivision

VMHvl, Ventromedial hypothalamus, ventrolateral subdivision

1 CHAPTER ONE: INTRODUCTION

1.1 Mood Disorders: Anxiety and Depression

Anxiety and depression are the most common psychiatric conditions, with a lifetime risk of 15-20% (Kessler et al., 2005a; Reul and Holsboer, 2002) and a cost to the United States of $113 billion dollars per year (Eaton et al., 2008). In both adolescents and adults, anxiety and depression are highly comorbid, occurring simultaneously in 85-90% of sufferers (de Jong et al., 2012; Kessler et al., 2005b; Ohayon and Schatzberg, 2010). These disorders also share a high degree of symptom overlap, including hypothalamic-pituitary-adrenal (HPA) axis dysregulation, behavioral changes, and alterations to both affect and sociability (Breslau, 2002; Enns et al., 2001). Commonalities among anxiety and depression prevalence and symptomology suggest these disorders share neurobiological underpinnings.

Females are at particular risk for mood disorders. Anxiety and depression are approximately twice as prevalent in women as men (Altemus, 2006; Nolen-Hoeksema, 1987). Some researchers have attributed the sex bias to disparities in self-reporting styles of men and women, or their likelihood to seek treatment (van Beek et al., 2012). Challenging this view is consistent reporting over the past 75 years that females are more often affected by anxiety and depression than males, a finding that holds across many nations, ethnicities, and cultures (Ellis et al., 2008; Kessler, 2003; Nolen-Hoeksema, 1987; Weissman and Klerman, 1977). While female bias in mood disorder prevalence is among the most robust observations in psychiatry, the origin of this sex difference remains unknown. Elucidation of the developmental origins of this sex difference is key to the advancement of mood disorder diagnosis and treatment.

Lifetime mood disorder prevalence among the preadolescent population is 1%, with such conditions affecting males and females under age 12 equally (Emslie et al., 2005). A dramatic rise in mood disorder rates is seen during adolescence reaching as high as 17-25% by the close of adolescence (Andersen and Teicher, 2008; Kessler et al., 2001; Nolen-Hoeksema and Girgus, 1994; Terrance et al., 2002). Nested within this adolescent rise in risk is another well-reported psychiatric finding, the concurrent emergence of female-biased mood disorder prevalence with the onset of puberty (Angold and Costello, 2006; Nolen-Hoeksema and Girgus, 1994). As mood disorder incidence rises among adolescent females, clinical depression becomes the most predominant psychological condition affecting this group (Bearman et al., 2003; MacPhee and Andrews, 2006). Taken together, these findings highlight the consistent and robust nature of female-bias in mood disorders, and point to an influential role of pubertal gonadal hormones on its development during adolescence.

1.1.1 Sexual differentiation and the development of "female risk"

The process of sexual differentiation includes the establishment of biological sex differences in the brain and behavior. This process occurs predominantly through widespread effects of gonadal hormones acting on target tissues that express their respective receptors (Blakemore et al., 2010; Sisk and Foster, 2004). Sexual differentiation of the brain involves the sex-specific organization of neural substrates by perinatal testosterone, and then subsequent activation of the already-organized brain by gonadal hormones after puberty (Phoenix et al., 1959; Schwarz and McCarthy, 2008). Likewise, sexual differentiation of the HPA axis is prenatally organized, and then activated by circulating pubertal and adult hormones (Seale et al., 2005). Sex differences are abundant within neurocircuits that process stress and emotion, and are thus investigated here as potential neural mechanisms contributing to female mood disorder

susceptibility. Moreover, because puberty coincides with increased mood disorder onset, biological mechanisms of sexual differentiation may also bring about sex-specific mood disorder risk.

1.2 Early-Life Stress Increases Risk of Mood Disorder Development

Anxiety and depression are "stress-related" mood disorders, which are brought on or exacerbated by stressful life events (Chapman et al., 2004; Duman and Monteggia, 2006; Kendler et al., 2003). The majority of depressive episodes are preceded by stressful life events (Hammen, 2005; Heim et al., 2008; Monroe et al., 2007; Young and Altemus, 2004), and traumatic stressors induce psychological symptoms and disorders more frequently than common stressors (Clum et al., 2001; Di Leone et al., 2013).

Adverse experience during early life is the most prominent environmental risk factor promoting the development of anxiety and depression (Edwards et al., 2003; Kaufman et al., 2000; Kessler et al., 2005b; Nolen-Hoeksema and Girgus, 1994; Penza et al., 2003). Early life adversity encompasses a broad range of experiences including physical abuse, sexual abuse, parental neglect, and bullying. Experiencing abuse, neglect, or parental loss during childhood is associated with increased mood disorder susceptibility (Bishop et al., 2014; Heim et al., 2000; Kendler et al., 1992; Martins et al., 2011), tripling the risk of depression later in life (Wise et al., 2001). A history of childhood maltreatment also advances depression onset in adulthood by five years (Lenze et al., 2008; Wainwright and Surtees, 2002; Widom et al., 2007), and maltreatment severity correlates with that of depression symptoms (De Bellis et al., 1999a; Green et al., 2010; Martins et al., 2011). Moreover, among individuals with anxiety and depression, those exposed to maltreatment during early life exhibit increased disorder comorbidity as well as suicide risk

relative to those without a history of maltreatment (Dube et al., 2001; Teicher and Samson, 2013a).

Adolescence is a developmental period during which chronic stress exposure may produce especially detrimental effects. The maturational epoch of adolescence spans the time between childhood and early adulthood, encompassing puberty, when physical, psychological, and social changes rearrange the brain and behavior in order to promote the development of independence and reproductive success (Blakemore et al., 2010; Sisk and Foster, 2004; Sisk and Zehr, 2005; Spear, 2000). However, heightened experience-dependent plasticity during adolescence may be conducive to disadvantageous neural changes following exposure to early life adversity, including maladaptive responses to future environmental challenges (Mccormick and Mathews, 2010; Romeo and Mcewen, 2006; Sisk and Foster, 2004).

During adolescence, brain development is still an active process. Because the adolescent brain is essentially underdeveloped, exposure to physical, sexual, or emotional maltreatment could induce particularly deleterious alterations in the brain and behavior. Beneficial processes are likewise occurring during this time, however, that are necessary for the development and fortification of the adult-like state. Pubertal hormones mediate the progressive maturation of brain regions including the hypothalamus, amygdala, hippocampus, nucleus accumbens, and sensory areas, which are especially important in the promotion of puberty-to-adult shifts in reproduction, sensory processing, attention, and motivation behaviors (reviewed in (Blakemore et al., 2010)).

The adolescent period is accompanied by changes in the social environment. In order to adapt to these transitions, the pubertal hormone-mediated maturation of the limbic and prefrontal brain regions facilitates associations between emotional elements and social stimuli (Bishop et

al., 2014; Nelson et al., 2005). Thus, adolescence is a transitional period comprising numerous internal and environmental changes, and increased vulnerability to stress-related psychopathologies. Additionally, characteristic internal (e.g., puberty, cognitive processing) and external (e.g., increased social interactions, self-dependence) changes in adolescence have been associated with increased risk of developing emotional problems, such as negative affect, emotional reactivity, and symptom internalization, particularly in females (Arnett et al., 1999; Larson and Ham 1993), yet it is unclear whether such emotional problems enhance female mood disorder risk, or vise versa.

The neuroendocrine stress response is still developing during adolescence. In rats, HPA axis activity following psychogenic stress differs between adolescents and adults in its magnitude and time-course, with adolescents exhibiting higher and more prolonged stress hormone levels than adults (Cruz et al., 2008; Goldman et al., 1973; Romeo et al., 2006). In humans, basal and stress-evoked cortisol levels gradually increase throughout adolescent development as neuroendocrine functioning matures to adult status (Clare et al., 2004; Gunnar et al., 2009; Richard et al., 2003; Walker et al., 2001). Increased responsivity of the HPA axis may render adolescents particularly vulnerable to harmful effects of chronic stress. Because HPA axis circuitry and feedback are immature before puberty, early-life and adult exposure to chronic stress may have different neural and behavioral consequences such that early-life stress more readily promotes lasting neuroendocrine alterations, while effects of adult stress tend to be more transient (Lupien et al., 2009; McEwen, 1999; Nelson et al., 2005).

1.3 Stress: Neuroendocrine Responses and Neural Substrates

Stress is the response of an organism to environmental challenges. Hans Selye originally defined the biological concept of stress as “the non-specific response of the body” to demands

placed upon it (Selye, 1946). Selye's General Adaptation Syndrome, which he later renamed the stress response, consisted of three phases: the initial alarm phase or fight-or-flight reaction, the resistance phase of adaptation to continued stressor exposure, and the exhaustion phase, when the body can no longer adapt to stressor continuance (Selye, 1951). Stressors vary widely by features such as type (e.g., physical/emotional, social/non-social), duration (e.g., transient/chronic), degree (e.g., mild/traumatic), and timing (e.g., prenatal/postnatal, adolescence/adulthood). Specific mechanisms of stress processing in the brain likewise vary in response to stressor-specific characteristics, for example, distinct stressors recruit distinct circuits comprising distinct brain region assemblies. Additionally, neuroendocrine, behavioral, and cognitive components of the stress responses vary in response to specific stressor features, as well as to more context-dependent variables such as expectation, biological sex, and age.

1.3.1 Hypothalamic-pituitary-adrenal axis response to stress

The HPA axis is the neuroendocrine system that responds to stressful stimuli. In both humans and laboratory rodents, information concerning perturbations in an organism's internal milieu or external environment is projected to the paraventricular nucleus of the hypothalamus (PVN), the chief initiator of HPA axis activity. From there, parvocellular neurosecretory cells of the PVN release corticotropin-releasing factor (CRF) into the hypophysial portal vessels, which drain to the anterior pituitary gland and stimulate adrenocorticotropic hormone (ACTH) release into circulating systemic blood (Dunn and Berridge, 1990). ACTH stimulates the release of glucocorticoids (e.g., cortisol in humans and corticosterone in rodents) from the adrenal cortex and catecholamines (e.g., adrenaline and norepinephrine) from the adrenal medulla (Axelrod and Reisine, 1984; de Kloet, 1984; Mccormick et al., 2010). Glucocorticoids interact with two receptor types, lower affinity glucocorticoid receptors (GRs) and high-affinity mineralocorticoid

receptors (MRs) (Ahima and Harlan, 1990; Aronsson et al., 1988; de Kloet, 1984; Morimoto et al., 1996; Reul and Kloet, 1985). Glucocorticoids have many targets throughout the peripheral and central nervous systems, where they interact with GRs to redirect energy resources (e.g., mobilize glucose stores, suppress immune and reproductive function), and promote adaptation and recovery following the stressor (Herman et al., 2003).

The regulation of HPA axis activity occurs primarily through a negative feedback mechanism that lowers stress-evoked elevations in circulating glucocorticoid levels (Abe and Critchlow, 1980; Tsigos and Chrousos, 2002). Under basal conditions, the HPA axis is kept in check by glucocorticoids acting on MRs in the hippocampus (Juruena et al., 2013). During times of stress, high glucocorticoid levels occupy both GRs and MRs in the PVN, anterior pituitary and, to a lesser extent, the hippocampus and prefrontal cortex (PFC), inhibiting CRF release from the PVN and lowering glucocorticoid levels (Herman et al., 2002; Herman et al., 1996; Jessica et al., 2013a; Juruena, 2014; Laryea et al., 2012; Plotsky et al., 1993; van Haarst et al., 1997). This self-regulation of glucocorticoid levels is thought to protect the brain from detrimental effects of chronic stress exposure (Handa and Weiser, 2014; McEwen, 2007).

HPA axis negative feedback occurs in two phases. Fast HPA feedback rapidly decreases stress hormone levels and neural activity via glucocorticoid signaling in the PVN, while delayed HPA feedback terminates HPA activity by glucocorticoid signaling in forebrain areas (e.g., hippocampus, PFC) (Joels et al., 2013; Myers, 2012). Together, fast and delayed HPA feedback coordinate and support efficacious adaptations to stress via distinct cellular mechanisms that regulate HPA function across multiple neural locations and time points (Joels et al., 2013; Myers, 2012).

1.3.2 Sex differences in the HPA axis

Sex differences present throughout the stress-responsive brain may facilitate sex-specific perception, processing, and responding to stressors. Basal and stress-evoked stress hormone levels, as well as the amplitude and frequency of their pulsatility, are higher in female than in male rats (Critchlow et al., 1963; Figueiredo et al., 2007; Kitay, 1961; Seale et al., 2005; Young et al., 2007). Stress-induced neural activation of the PVN and circulating ACTH levels are higher in adult females and lower in adult males, than in prepubertal male or female rats, which do not differ from one another (Viau et al., 2005). In a study using forebrain cortico-limbic-specific GR knockout mice (FBGRKO), Solomon et al. found that male knockouts displayed elevated depression-like behavior, as well as basal, and stress-evoked corticosterone levels compared to controls (Solomon et al., 2012). Neither depression-like behavior nor corticosterone levels in FBGRKO female mice differed from controls, suggesting that GRs in forebrain regions modulate HPA axis negative feedback to a greater extent in males (Solomon et al., 2012). Also in FBGRKO mice, females but not males showed decreased anxiety-like behavior, indicating sex-specific influences on mood disorder-like behavior.

Reciprocally, gonadal hormones can also act on neural substrates of stress to remodel aspects of physiology and behavior. For example, estradiol increases CRF gene expression (Bohler et al., 1990). Ovariectomized adult female rats show blunted ACTH and corticosterone secretion after stress and this effect is reversed by estradiol treatment (Burgess and Handa, 1992; Vamvakopoulos and Chrousos, 1993; Weiser and Handa, 2009). Similar studies reveal that testosterone exaggerates corticosterone levels and CRF-immunoreactivity in the PVN of males (Handa et al., 1994b; Viau et al., 2001). In the bed nucleus of the stria terminalis (BNST), an area highly implicated in anxiety pathology, androgen depletion by gonadectomy reduces CRF mRNA levels, and testosterone replacement restores them (Viau et al., 2001). Together, such

findings suggest that under normal physiological conditions, ovarian hormones may augment HPA axis responsivity, while testosterone may reduce it.

Although few studies have investigated sex differences in subjective and emotional aspects of the stress response, those that report these measures find women more reactive to stress than men. Women report higher subjective distress levels, as well as higher symptom severity relative to males (Angst and Dobler Mikola, 1984; Frank et al., 1988; Fujita et al., 1991; Grossman and Wood, 1993). Most recently, women show higher EEG response amplitude to negative emotional stimuli than men, suggesting greater emotional reactivity (Gardener et al., 2013).

1.3.3 Neural processing of stress

The perception, processing, and neuroendocrine response to stress are largely controlled by a group of highly interconnected cortical and limbic brain regions, including the PFC, hippocampus, PVN, BNST, and amygdala. This group orchestrates neuroendocrine stress responses via HPA axis activation, which is structured to mediate the successful adaptation and future responding to environmental challenges (Deppermann et al., 2014; Diorio et al., 1993; Jessica et al., 2013a; van Haarst et al., 1997). Upon stressor exposure, neural activity increases in stress-responsive brain regions, inducing the transmission and convergence of stressor-specific signals to the PVN, thereby stimulating HPA axis activity (Herman et al., 2005). Physical stressors (e.g., pain, thirst), in large part, activate the HPA axis via direct projections to the PVN from the BNST, peri-PVN zone, hypothalamus, and brainstem (Herman et al., 2003). Psychogenic stressors (e.g., social isolation, restraint), especially those of a social nature (e.g., social subjugation, social instability), activate the HPA axis by way of indirect projections to the PVN from the BNST, lateral septum (LS), medial prefrontal cortex (mPFC), and amygdala

subnuclei (Herman et al., 2003; Herman et al., 2005; Roland and Sawchenko, 1993). Gonadal and stress hormone receptor distributions are highly overlapping in brain regions that process stress and emotion, including the PFC, hippocampus, amygdala, and BNST (Handa et al., 1994a; Viau and Meaney, 1996; Viau et al., 2001). High sex hormone sensitivity among these PVN inputs reflects the biological importance of sex-specific modulation of the HPA axis.

The amygdala is quickly and robustly activated by stressful and emotional stimuli and contributes to behavioral responses to stress such as arousal and fear (Davis et al., 1997; Phelps and LeDoux, 2005). Furthermore, it plays a major role in the coordination of neuroendocrine, emotional, cognitive, autonomic, and behavioral aspects of stress responsivity. The amygdala's widespread ability to coordinate may reflect its interconnectivity with HPA modulators, the peri-PVN and BNST (Canteras et al., 1995). Stress-responsive regions among cortico-limbic circuits also modulate the perception and expression of emotion, as well as emotional memory. Significant anatomical overlap between neurocircuitries of stress and emotion suggests that emotional regulation of stress may interact with sex to affect mood disorder risk (Palazidou, 2012). Moreover, cortico-limbic regions are robustly and intricately interconnected. For instance, the amygdala shares reciprocal projections with the PFC and hippocampus, establishing it as a site of complex signal integration, which could result in finely tuned coordination of emotional responses. Indeed, such integration may be the neural mechanism by which the amygdala modulates performance of complex emotional tasks such as emotional memory consolidation and social recognition (Adolphs et al., 2002; Adolphs et al., 1999; McGaugh, 2004). When a stressor is perceived, afferent signals about the stressor the travel toward the amygdala from other stress-responsive areas, including the brainstem, hypothalamus, septum, cingulate cortex, and hippocampus (Herman et al., 2005). Subsequently, efferent projections carrying information

about the stress response from the amygdala, to other stress-responsive areas, including the brainstem, hypothalamus, septum, insula, and PFC, each of which further influences autonomic, endocrine, and behavioral aspects of stress responsivity (Herman et al., 2005).

Important to this body of work are the distinct amygdaloid subnuclei and their functional roles in stress processing. The amygdala's ability to rapidly respond to environmental threats is largely due to its ability to receive and integrate sensory information. The immediate transmission of sensory signals occurs via direct projections from sensory cortices to the basolateral subnucleus (BLA), which ascribes emotional significance to sensory stimuli (Pitkänen et al., 1997; Stamatakis et al., 2014). The BLA projects to the central subnucleus (CeA), which receives and processes viscerosensory details accompanying physical stress, and influences autonomic and emotional aspects of the stress response. Additionally, the CeA is the major output nucleus of the amygdala, and a main extra-hypothalamic site of CRF synthesis and stress-induced release in the brain, second only to the PVN (Li et al., 1996; Merlo Pich et al., 1995; Pitkänen et al., 1997). The medial subnucleus of the amygdala (MeA) is the principal processing center of psychogenic stressors such as social defeat, social recognition, sexual attraction, and sex behavior. (Dielenberg and McGregor, 2001; Ferguson et al., 2001; Kelliher et al., 1999; Martinez et al., 2002; Wersinger et al., 1993). The posterior MeA is further subdivided into the posterodorsal (MePD) and posteroventral (MePV) subnuclei, each of which uniquely participates in threat detection and defensive behavior (Pardo-Bellver et al., 2012). Hormone sensitivity is a characteristic feature of the amygdala; gonadal hormones as well as CRF receptor expression levels are particularly high in the MeA, especially in posterior subnuclei (De Souza et al., 1985; Mizukami et al., 1983). Furthermore, subnuclei of the amygdala exhibit various sexual dimorphisms such as in gonadal hormone sensitivity, synaptic connectivity, dendrite

morphology, and regional volume (Cooke et al., 2007b; Cooke and Woolley, 2005b; Simerly, 1993). Altogether, the amygdala's widespread neural connectivity, gonadal hormone sensitivity, responsivity to social stimuli, and neural processing of stress and emotion point to this structure, and specifically the MeA, as a potential neural substrate for the sex-specific processing of chronic early life stress.

1.4 Extended Role of the CRF System in the Stress Response

Wylie Vale and colleagues first isolated CRF nearly 30 years after Selye suspected the existence of a molecule in the brain responsible for pituitary corticotropin release (Vale et al., 1981). Subsequently, investigations have established the intimate role of CRF and its family of related molecules in stress processing, and strongly implicated it in mood disorder-related neuropathology. The CRF system consists of four ligands, CRF and three peptides belonging to the urocortin family (Ucn1, Ucn2, and Ucn3), and two CRF receptor subtypes (CRF1 and CRF2). CRF and the urocortin family of ligands bind with varying affinities to CRF receptors. CRF binds CRF1 with high affinity, while the urocortins bind to CRF2 with high affinity and CRF1 with low affinity (Heinrichs et al., 1997; Reyes et al., 2001). Distribution patterns of CRF receptors in the brain are discrete and mostly non-overlapping. CRF1 is largely expressed in sensory processing and motor control areas such as the neocortex, cerebellum, and thalamus, as well as in subcortical regions including the amygdala, hippocampus, and anterior pituitary (De Souza et al., 1985; Reul and Holsboer, 2002). CRF2 is predominantly found in subcortical and limbic structures such as the lateral septum, hippocampus, amygdala, BNST, and hypothalamus (Chalmers et al., 1995; Refojo and Holsboer, 2009; Reul and Holsboer, 2002).

CRF is a particularly effective stress-signaling molecule due to its ability to function as a neurohormone in the HPA axis, as well as a neurotransmitter, allowing widespread and

anatomically diverse stress effects throughout the brain. Beyond that, CRF coordinates autonomic and behavioral aspects of the stress response (Owens and Nemeroff, 1991; Raux-Demay and Girard, 1985; Reul and Holsboer, 2002; Vazquez et al., 2003). The PVN and CeA are the major sites of CRF synthesis in the brain. Furthermore, CRF neurotransmission, unlike that of most metabotropic neurotransmitters, activates receptors in a paracrine fashion, simultaneously activating many neurons, potentially promoting widespread responses to stressors that are particularly intense or traumatic.

Central administration of CRF in rats results in the stimulation of hypothalamic and limbic brain regions that influence the HPA axis, activates the autonomic nervous system, increases stress-related behaviors such as anxiety, fear, despair, and emotionality, and decreases food intake, sexual activity, exploration, and social interaction (Campbell et al., 2004; Dunn and Swiergiel, 2008; Imaki et al., 2001; Rivest et al., 1989; Sutton et al., 1982). Specifically, the activation of CRF1 receptors by CRF reliably activates the HPA axis and elevates circulating stress hormone levels (Arborelius et al., 1999; Heinrichs and Koob, 2004). CRF1 knockout mice exhibit reduced stress-induced ACTH and corticosterone release and adrenal medulla atrophy (Timpl et al., 1998). CRF2 knockout mice display hypersensitive corticosterone responses that are slower to recover from acute stress (Bale and Vale, 2003). Once thought to work in opposition to CRF1 receptors by decreasing HPA axis responses to stress, CRF2 receptors have since been observed to increase, decrease, and not affect HPA axis responsivity based on evidence from three separate lines of CRF2 knockout mice (Bale et al., 2000; Contarino et al., 2000; Coste et al., 2000; Jamieson et al., 2006). Mice lacking both CRF1 and CRF2 receptors exhibit severe HPA axis dysfunction consisting of elevated CRF levels in the PVN, blunted basal ACTH and corticosterone levels, and atrophy of the adrenal cortex (Bale et al., 2002). A similar

phenotype is observed in CRF1 deficiency alone, indicating that CRF2 cannot overcome the loss of CRF1 (Preil et al., 2001).

1.4.1 Sex differences in the CRF system

Some stress-sensitive neural targets of CRF receptor signaling are sexually dimorphic, making them candidates for mediating sex-specific stress effects in the brain. In rats, females typically express higher CRF levels than males in regions including the PVN, CeA, and the BNST (Iwasaki-Sekino et al., 2009; Sterrenburg et al., 2012; Victor et al., 2005). Recently, CRF receptor signaling was found to be sexually dimorphic at the sub-cellular level. In rat cortical neurons, CRF1 receptors more readily couple to stimulatory G proteins to increase cellular activity more in females than males (Bangasser et al., 2010; Bangasser and Valentino, 2012). Because CRF1 receptors stimulate the HPA axis, greater female CRF1 receptor activation could underlie dimorphic HPA axis sensitivity, rendering females more susceptible to stress-related psychopathologies. In stressed adult males rats, but not females, cortical CRF_1 receptors interact with βarrestin2, which facilitates receptor internalization and inhibits G protein-coupled second messenger activity. This suggests a mechanism by which stress induces CRF1 receptor desensitization more in males than females, rendering them less sensitive to CRF and able to recover faster from stress. The potential contribution of molecular level sex differences in CRF receptor function to mood disorder development have not been identified.

Additionally, chronic mild variable stress has been shown to induce sex-specific methylation of the CRF gene promoter, decreasing methylation in the male and female BNST, while increasing methylation in the PVN of females only (Sterrenburg et al., 2012). This finding may reflect a priming effect in females whereby experiencing one stressor alters the ability to mount a subsequent stress response. Urocortin 2 (Ucn 2) selectively binds and activates CRF2

and is expressed among stress-sensitive brain regions including the PVN, supraoptic and arcuate nuclei of the hypothalamus, and locus ceruleus (Reyes et al., 2001). Interestingly, Ucn 2 null mice sex-specifically express stress-related phenotypes such that females, but not males, exhibit elevated basal ACTH and corticosterone levels and reduced depression-like behavior (Chen et al., 2006). Furthermore, although both male and female CRF2-lacking mice display increased depression-like behavior that decreases following treatment with the CRF1 antagonist antalarmin, and this decrease lasts significantly longer in females (Bale and Vale, 2003). Sexually dimorphic components of the CRF system may be neural substrates involved in sex-specific expression of mood disorder susceptibility.

1.4.2 Human studies: mood disorders are associated with CRF system abnormalities

Stress-related mood disorders are often associated with abnormalities in the HPA axis response to stress (Ehlert et al., 2001; Heim et al., 2000; Heim et al., 2002; Holsboer, 2001; Yehuda, 2001). Most commonly, mood disorder patients express CRF overactivity, attenuated negative feedback, and either hyper or hypocortisolism (Carroll et al., 2007; Gold and Chrousos, 2002; Parker et al., 2003; Paslakis et al., 2011). Initial experimental findings identified a subset of depressed patients displaying elevated cerebrospinal CRF levels (Nemeroff et al., 1984), an observation also reported for combat veterans suffering from PTSD (Bremner et al., 1997). More recent postmortem findings have reported elevated CRF levels in the brains of depressed patients, as well as in suicide victims (Merali et al., 2004). Similarly, disrupted CRF1 receptor expression may be associated with psychopathology. The same postmortem study reporting elevated CRF levels in the brains of suicide victims found a reduction in CRFR1 transcripts (Merali et al., 2004), a finding replicated in additional studies (de Kloet et al., 2005b; Regev and Baram, 2014).

1.5 Animal Assays of Mood Disorder-Like Pathology

Comprehensive animal models of mood disorders do not yet exist, as researchers are unsure how to capture complete disorder-specific biopsychosocial profiles in laboratory animals (McKinney, 2001). Nevertheless, meaningful investigations into mood disorder-like pathology have used well-validated animal models of psychiatric condition-specific traits and behaviors (Pawlak et al., 2008). Rodent assays of mood disorder-like psychopathology can be broadly classified into two categories: those that measure baseline mood disorder-like behaviors, and those that measure evoked mood disorder-like behaviors following exposure to a stressor (Seney and Sibille, 2014). Baseline assays are thought to reflect mood disorder predisposition, while stress-evoked assays aim to measure stress-induced mood disorder-like pathology, as stress is a common trigger of stress-related psychopathologies such as anxiety and depression (Chapman et al., 2004; Duman and Monteggia, 2006; Kendler et al., 2003).

High conservation of threat detection neural circuitry among mammals allows for laboratory investigations into pathological anxiety-like behavior using various rodent species (Dias et al., 2013). Animal models of anxiety-like behavior evaluate specific symptomology of human anxiety such as conflict anxiety (Gray and McNaughton, 1996; McNaughton and Corr, 2004). Assays of conflict anxiety assess the relative expression of a rodent's inherent approach and avoidance behaviors, where approach is a proxy for exploratory behavior, and avoidance is a proxy for anxiety-like behavior (Denenberg, 1969).

Commonly used behavioral paradigms of anxiety-like behavior include the open field test (OF), elevated plus maze (EPM), and social interaction test (SIT) (reviewed in (McCormick and Green, 2013)). The OF is a highly reliable test of fear and anxiety that induces moderate anxiety by inescapable exposure to novelty (Belzung, 1992; Brown, 2002), and has been widely used since its conception 80 years ago (Denenberg, 1969; Stanford, 2007). In this test, an animal is

placed in a large open arena, and the ratio of time spent in the arena's center versus its periphery is quantified (Nestler and Hyman, 2010; Treit and Fundytus, 1988). Here, the relative safety of the periphery is pitted against the novelty of venturing into the open center, and exploratory entries into the arena's unprotected center reflects less-anxious behavior, while more time in the periphery corresponds to higher anxiety-like behavior. The EPM is a raised, plus-shaped maze possessing two arms with walls, and two without. Here, anxiety-like behavior is assessed by the ratio of time spent in the open arms (exploration) versus the closed arms (avoidance) (Nestler and Hyman, 2010; Wall and Messier, 2001). Rodents tend to prefer dark, protective spaces; therefore, entry into the open arms reflects less-anxious behavior, whereas more time spent in the closed arms reflects higher anxiety-like levels.

Commonly used behavioral tests of depression-like behavior in rodents include the forced swim test (FST) and the sucrose preference test (reviewed in (Razafsha et al., 2013)). In the FST, a rodent is placed in an inescapable cylindrical water tank, and the latency to become immobile and the total duration spent immobile are measured, as they are considered reflective measures of behavioral despair (Porsolt et al., 1978; Porsolt et al., 1977). Long immobility latencies and short immobility durations indicate low depression-like behavior. The FST has been extensively validated as a screening tool for antidepressants, as they reliably decrease immobility behavior (Cryan et al., 2005). The sucrose preference test measures hedonic responses to pleasurable stimuli. During this assay a rodent is given *ad libitum* access to tap and sucrose water, and sucrose consumption is quantified (Grippo et al., 2003; Loas, 1996). Decreased or minimal sucrose consumption is considered indicative of anhedonia. Like the FST, antidepressant treatment increases sucrose water intake, reducing anhedonia in rodents (Willner, 1990).

Anxiety and depression possess distinct, yet partially overlapping, symptomologies (Clark and Watson, 1991). The social interaction test (SIT), is used to measure sociability, which is reduced in both anxiety and depression (Kimberley and Mary Jane, 2006). During the SIT, an experimental animal is typically paired with an age- and sex-matched conspecific in a neutral cage, and the frequency and duration of social interactions are quantified. Investigatory approaches, grooming, play, and aggression, are considered proxies for sociability. On the other hand, low expression of these behaviors reflect social withdrawal, indicating an anxiety and depression-like behavioral phenotype (Flint and Shifman, 2008).

Stress-evoked behavioral assays explore the effects of stress on mood disorder-like pathology. Stressors used may be nonsocial, such as predator and chronic variable stress, or social, such as social instability or social defeat stress. Predator stress exposes a rat to cat odor, while chronic variable stress measures the combined effect of multiple nonsocial stressors (e.g., forced swim, restraint, etc.). In humans, mood disorder development is predominantly fostered by stressful experiences that are social in nature (Brown, 2002; Buwalda et al., 2005). Social instability stress involves the frequent switching of rodent cage mates such that stable social structures cannot form. Chronic social defeat stress typically utilizes the resident-intruder paradigm, during which an intruder rodent is introduced into the home cage of a resident, typically a larger and more aggressive age-matched conspecific male, which then displays aggression toward the intruder until social dominance/submission is established (Kudryavtseva et al., 1991; Miczek, 1979).

Although social defeat stress is more reminiscent of human early-life maltreatment than other stress paradigms, empirical limitations create challenges when using this paradigm. Because adult rats do not readily display aggression toward juveniles, only a subset of social

defeat stress models deliver stress during adolescence, despite the highly social nature of adolescent stressors and potentially enhanced stress sensitivity during this developmental period (Becker et al., 2005; Holder and Blaustein, 2014). Another limitation of current adolescent social defeat models is that, despite the female-biased prevalence in stress-related mood disorders, tests are predominantly conducted using only male subjects, as resident females do not display male-like aggression levels (Kendler et al., 2002; Palanza et al., 2001).

In order to test the effect of chronic adolescent social defeat on sex-specific mood disorder-like pathology in adulthood, we developed a novel social defeat paradigm that occurs during adolescence and includes male and female subjects, bridging specific gaps in early-life stress research. Juvenile Social Subjugation (JSS), is a variant of the resident-intruder paradigm (Miczek, 1979), that uses adult male residents, and male and female adolescent (P28) intruders to model early-life stressors inflicted by an adult, such as parental neglect or maltreatment. The JSS paradigm consists of daily exposure of adolescent rats to an aggressive adult male. Once adult, intruders are tested in a number of well-validated assays that quantify mood disorder-like characteristics (e.g., OF, EPM, FST, SIT), including anxiety- and depression-like behaviors, and neuroendocrine measures of HPA axis function.

1.6 Dissertation Overview

The overarching goal of this dissertation is to examine long-lasting and sexually dimorphic mood disorder-like changes in behavior and stress responsivity following chronic adolescent social stress, and their underlying neural mechanisms. To address this, I will discuss investigations surrounding the following research questions: (1) Does chronic JSS exposure induce sexually dimorphic expression of anxiety and depression-like behaviors and HPA axis dysfunction in adulthood? (2) Is JSS differentially processed by the male and female adolescent

brain? (3) Are neural substrates of stress, namely the CRF receptor system, sex-specifically expressed across development? Taken together, these studies aim to elucidate sex-specific neural mechanisms by which early-life stress may induce sex-specific vulnerabilities to stress-related psychiatric disorders.

2 CHAPTER TWO: JUVENILE SOCIAL SUBJUGATION INDUCES A SEX-SPECIFIC PATTERN OF ANXIETY AND DEPRESSION-LIKE BEHAVIORS IN ADULT RATS

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2.1 Abstract

Child abuse is the most significant environmental risk factor for the development of mood disorders, which occur twice as frequently in women as in men. To determine whether juvenile social subjugation (JSS) of rats induces mood disorder-like symptoms, we exposed 28 day-old male and female rats to daily aggressive acts from aggressive male residents. Each rat received pins, kicks, and dominance postures from the resident for 10 minutes per day for 10 days. When the rats were adults, we tested their anxiety- and depression-like behaviors. In addition, we measured circulating basal and stress-evoked corticosterone (CORT) levels, and weighed the adrenal glands. Although the amount of JSS was indistinguishable between males and females, females were nonetheless more severely affected by the experience. Subjugated females became immobile more quickly during forced swim tests, and made fewer investigatory approaches during the social interaction test than control females. Juvenile social subjugation

increased closed arm time in the elevated plus maze of males and females, but the effect of social subjugation was greater in females. Finally, stress-evoked CORT levels were significantly higher, and adrenal gland weights were significantly heavier, in subjugated females relative to their controls and to subjugated males. Our results demonstrate that JSS increases depression- and anxiety-like behaviors and sensitizes the stress response system in a sex-specific manner.

2.2 Introduction

Anxiety and depression are the most common stress-related mood disorders. They affect approximately 20% of Americans and are frequently co-morbid with one another (Murphy et al., 2004; Penza et al., 2003). The rate of depression and anxiety is approximately twice as high in women as it is in men, a sex difference that emerges during puberty (Altemus, 2006; Ellis et al., 2008; Weissman and Klerman, 1977). The origin of this sex difference in mood disorders is unknown, although it has been suggested that this may be due to an increased susceptibility of females to stress (Altemus, 2006; Becker et al., 2007).

Adverse early experience is a major risk factor for anxiety and depression in adulthood (Dube et al., 2001; Edwards et al., 2003; Felitti et al., 1998; Heim and Nemeroff, 2002; Penza et al., 2003). Early life adversity encompasses a broad range of experiences including physical and sexual abuse, and bullying (Nansel et al., 2001). Mood disorders in adulthood are far more likely to occur if the patient has had a history of child abuse (Hawker and Boulton, 2000; Kessler, 1997). Furthermore, mood disorder risk is positively related to the diversity of types of childhood maltreatment (Edwards et al., 2003). Although boys and girls are victimized by abuse at approximately equal rates in the U.S. (2006), women with a history of childhood maltreatment are particularly susceptible to the development of affective disorders (Lenze et al., 2008). Physical and/or sexual abuse is associated with more intense emotional and physical symptoms

of depression, and women with such a history make more suicide attempts than women with mood disorders that were not abused as children (McCauley et al., 1997).

A common symptom of stress-related mood disorders is hypothalamic-pituitary-adrenal (HPA) axis dysregulation, evident in resistance to dexamethasone-induced suppression of cortisol release (Nemeroff and Evans, 1984), and in elevated cerebrospinal fluid levels of corticotrophin releasing factor (Nemeroff et al., 1984). Depressed women with a history of child abuse have higher stress-evoked cortisol than depressed women without such a history (Heim et al., 2000; Heim et al., 2008; Heim et al., 2002).

Social subjugation is a widely studied form of social stress in laboratory rodents and may be the most severe psychogenic stressor, as measured by sympathetic and HPA axis activation in the adult male rat (Koolhaas et al., 1997). As such, social subjugation reliably alters the emotional state of rodents and can induce mood disorder-like symptoms. For example, socially subjugated adult male rats show classic depression- and anxiety-like behaviors, including increased closed arm time in the elevated plus maze, anhedonia, weight loss, decreased sociability and chronically elevated CORT levels (Blanchard et al., 1995; Blanchard et al., 2001; Meerlo et al., 1996; Rygula et al., 2005). Furthermore, socially subjugated adult male hamsters display elevated CORT and intense fear during future physical and non-physical social interactions (Huhman, 2006; Huhman et al., 1991; Huhman et al., 1992) while socially subjugated juvenile male hamsters exhibit increased aggression toward smaller conspecifics (Delville et al., 1998; Wommack et al., 2003).

Puberty is an important developmental epoch because sex differences in mood disorder prevalence emerge during this time (Altemus, 2006; Cyranowski et al., 2000; Ge et al., 1994), and because stress responsiveness is prolonged in prepubertal animals relative to adults (Romeo

et al., 2004a; Romeo et al., 2004b). This may render the developing brain more vulnerable to insults and increase the risk of stress-related mood disorders and cognitive deficits (Hodes and Shors, 2005; Lupien et al., 2005; Mccormick et al., 2010).

Although attention to puberty and adolescence has been increasing of late (e.g., (Mccormick et al., 2010), there have been comparatively few studies of social stress that have used juvenile rats. And in spite of the clear relationship between gender, puberty, stress, and mood disorder risk, even fewer animal studies of mood disorders have included females. In one such experiment, one hour of social isolation during adolescence coupled with daily cage partner changes was associated with sex-specific changes in the HPA axis and in anxiety-like behaviors (Mccormick et al., 2005). The same regimen also enhanced sensitization to psychomotor stimulants in females, but not in males (McCormick et al., 2004). These findings indicate that adverse experience during puberty can have sex-specific effects on emotion, the HPA axis, and possibly the mesocorticolimbic dopaminergic system. It is unknown, however, what impact juvenile social subjugation has on mood disorder-like behaviors. This is an essential question to answer because of the profound effect child abuse has on the development of mood disorders later in life. Thus, to determine whether juvenile social subjugation (JSS) during adolescence affects mood disorder-like behaviors and HPA axis function, we subjected male and female juvenile rats to repeated physical subjugation from an aggressive adult male, and then assessed their anxiety- and depression-like behaviors in adulthood.

2.3 Materials and Methods

2.3.1 Screening Resident Aggressors

Four weeks prior to the arrival of each cohort of juveniles, 15 postnatal day 60 (P60) male Long Evans rats were obtained from Charles River and housed individually. To increase

the territoriality of these males, cage changes were reduced to once per week. Three weeks prior to the JSS procedure, the males were screened to identify those that were reliably aggressive toward male and female juvenile rats. In the screening procedure, a P28 male or female rat was introduced into the male's home cage and the number of aggressive acts toward the stimulus juvenile was recorded. From the original set of 15 male rats, the five most aggressive males were selected to be the resident aggressors (RAs). In preliminary studies, we observed that housing the RA with a sexually receptive female increased aggressiveness. Thus, each RA was paired with an adult ovariectomized female that, twice per week, received subcutaneous injections of estradiol (0.1 mg) followed by progesterone (0.5 mg) 24 hours later.

The RAs and their cage mates were housed in modified cages (53 x 29 x 20 cm) that allowed the insertion of a transparent and perforated partition that created a compartment ~ one third the size of the overall cage.

All animals were given *ad libitum* access to food and water and were housed on a 12-h reversed light cycle (lights off 0700 h) in a temperature-controlled (23o C) animal facility. All procedures were previously approved by the Georgia State University Institutional Animal Care and Use Committee and met the standards outlined in the *N.I.H. Guide for the Use of Laboratory Animals.*

2.3.2 Juvenile Social Subjugation

P21 Long Evans rats were obtained from Charles River in two cohorts of ten males and ten females each. Subjects were housed individually in standard laboratory rat cages (45 x 23 x 20 cm) with corncob bedding. Rats were left undisturbed for one week except for feeding and cage changes. Six days after their arrival at the GSU vivarium, juveniles were randomly assigned to handled control (HC) or juvenile social subjugation (JSS) groups. We then used a procedure

adapted from the original resident-intruder paradigm of (Miczek 1979). To begin the JSS procedure, the RA's female cage mate was removed and the partition inserted. A juvenile was then introduced into the smaller compartment of the RA's cage. After five minutes, the partition was removed and the juvenile and resident were allowed to interact for 10 minutes. The partition was then replaced, and the juvenile was returned to the smaller compartment for another five min, after which it was returned to its home cage. The adult female was then returned to the RA's cage. No juvenile was exposed to the same resident more than twice in 10 days. Episodes were videotaped, and an investigator later scored the number and types of aggressive acts (pins, rear paw kicks, and dominance postures) during each JSS episode. Pins were scored when an RA immobilized of the juvenile in a supine position. Rear paw kicks were scored when the RA used its rear paws to kick the intruder. Dominance postures were scored when the RA immobilized the juvenile with its front paws, causing it to freeze in a prone position.

HC rats were treated to identical procedures as the JSS rats, including two 5 min periods behind the partition and one 10 min period exploring the cage, except that the rats were placed into a clean cage containing only fresh bedding.

The JSS and HC procedures occurred once daily over the course of two work weeks between 1000 h and 1400 h. Thus, each rat received a total of 10 JSS or 10 HC experiences between the ages of P28 and P41.

2.3.3 Behavioral Testing

Following the final JSS or HC experience, rats were left undisturbed, aside from bi-weekly cage changes, for three weeks. Behavioral tests were initiated when the rats were P63 and were conducted during the dark phase of the animal's light cycle between 1000 and 1300 h

(Fig. 1). All tests were video recorded and later manually coded using Noldus Observer (9.0) or automatically with Noldus Ethovision XT (7.0).

First, an open-field test was conducted on all rats to assess locomotor activity and exploratory behavior. Then, to minimize potential sequence effects of the subsequent tests, the rats were randomly assigned to three groups (comprised of male and female JSS and HC rats) for behavioral testing, which was scheduled using a randomized block design. The rats were subjected to three tests over three weeks: Porsolt's forced swim test, the elevated plus maze, and a social interaction test. All behavioral tests were conducted under 25 watt red lighting except for the forced swim test, which occurred under indirect fluorescent lighting.

2.3.4 Estrous cycle phasing

Vaginal lavage was initiated seven days prior to behavioral testing and continued throughout the experiment. Lavages were performed daily between 0900 and 1000 h by gently lifting the tail, and inserting a smooth glass pipette containing 100 µL of 0.9% saline at 37ºC. The contents of the pipette were expelled, retrieved, and collected into a 12 well plate. To assess estrous cycle phase, cellular morphology of each lavage sample was inspected under 10X on each day of behavioral testing. Criteria for classifying each lavage sample as representative of a particular estrous cycle phase are as follows: estrous was identified by the predominant presence of cornified epithelial cells, proestrous was marked by nucleated epithelial cells, diestrous 1 consisted of predominantly leukocytes, and diestrous 2 included both leukocytes and large round anucleated cells (Becker, Arnold et al. 2005).

2.3.5 Open field

The open field arena was 90 cm2 with 40 cm walls. Lines on the floor of the open field created thirty-six 15 cm2 grid squares. Each animal was placed into the center of the arena and

allowed to explore for 10 min. After each trial, the animal was returned to its home cage and the floor and walls of the open field were cleaned with 70% ethanol.

The animal's behavior in the open field was recorded from a ceiling-mounted camera and tracked by Ethovision. The software counted line crosses and time in the center of the arena versus the periphery. The center of the arena was defined as the innermost 16 grid squares.

Forced Swim Test

A cylindrical tank (37 cm diameter x 50 cm deep) was used for the swim test arena. The container was filled with 25° C water leaving five cm between the waterline and top of the container. To begin the test, the rat was placed into the center of the pool. Following each swim, the rat was removed, dried with towels, and placed into a warm, dry cage. If an animal escaped from the tank, it was removed from the analysis (n=1). Each rat was required to swim for five minutes on three consecutive days.

Immediately after the first swim, blood was collected from the saphenous vein into a heparinized eppendorf tube. Samples were stored on ice, centrifuged at 15,000 rpm for five minutes, then blood plasma was decanted and stored at -20° C until determination of CORT levels.

Trials were video recorded from the ceiling and coded by two investigators, one of whom was blind to group membership. A rat was judged to have stopped swimming when it was immobile for ≥ two sec. Because rats are seldom truly immobile, the judgment was achieved by consensus between the two investigators. A third investigator, also blind to group membership, coded the rats' immobility time and swimming and climbing time. This investigator had achieved a ≥ 90% reliability with herself and the lead author before coding these behaviors.

2.3.6 Elevated Plus Maze

The elevated plus maze consisted of four stainless steel arms, each 10 cm wide and 110 cm long. Two arms were enclosed with 43 cm high stainless steel walls. The central zone was 10 cm2. Animals were placed onto the center of the maze and allowed to freely explore for five min. After each trial, the rat was returned to its home cage and the floor and walls of the maze were cleaned with 70% ethanol.

A ceiling-mounted video camera recorded each trial. A single investigator, blind to the subject's group membership, measured time spent in the closed and open arms as well as the number of entries into the close and open arms using Noldus Observer. Percent open and closed arm entries were calculated as the ratio of (number of entries into open or closed arms/total number of arm entries) x 100. Stretch attend behaviors were also coded and were counted when a rat stretched its head and two forepaws forward, out of a closed arm, and then returned to its original position. If a rat fell off the maze, its data were excluded (n=10).

2.3.7 Social Interaction Test

In this test, we again employed the resident-intruder test, but this time to measure sociability. The experimental rat served as the resident and an age- and sex-matched stimulus rat was introduced to the resident's homecage for ten minutes to determine the subject's reaction to an intruder. A cage, identical in dimension to the rat's home cage but with its floor removed was secured to the top of the resident's cage upside down, which allowed the rats to stand. After each trial, this was cleaned with 70% ethanol.

Two investigators, both of whom were blind to the group membership of the animals, coded the number of pins, rear paw kicks, dominance postures, as well as investigatory approaches by the resident toward the intruder. Investigatory approaches were identified when

the experimental animal approached the stimulus rat (the intruder) and sniffed the intruder's body, face, or ano-genital region. As a measure of locomotor activity, the number of times the experimental rat crossed the mid-line of the cage was counted. Inter- and intra-rater reliability for each parameter was ≥ 0.9.

2.3.8 Collection of trunk blood and organ collection

Three days after the final behavioral test at P83, and shortly after lights off in the vivarium (1700 h), rats were removed one-by-one from the vivarium, rapidly decapitated with a sharp guillotine, and trunk blood was collected. The blood was centrifuged and the plasma was decanted and stored at -20º until determination of basal CORT levels. The adrenal glands, thymus, and seminal vesicles were then removed and weighed.

2.3.9 Radioimmunoassay

Serum CORT levels in the rats were determined with radioimmunoassay, using a kit from MP Biomedicals (Cat # 07-120102). The linearity of dilution was used to validate the kit and yielded 91% recovery. The coefficient of variance was 5.5%. The correlation coefficient was 99%. The cross-reactivity of the kit with desoxycorticosterone and testosterone was 0.34% and 0.10%, respectively. The cross-reactivity with other related compounds was less than 0.05%.

Statistics

Using SPSS, and unless otherwise noted, analysis of variance (ANOVA) followed by post hoc tests was used to identify significant effects of juvenile social subjugation, time, or sex. Alpha was set to 0.05.

2.4 Results

2.4.1 No sex difference in the number of aggressive acts

We observed three types of aggressive acts during the JSS episodes: pins, rear paw kicks, and dominance postures. Pins occurred when the RA immobilized the juvenile in a supine position. Rear paw kicks occurred when the RA used its rear paws to kick the intruder. Dominance postures occurred when the RA immobilized the juvenile with its front paws, causing it to freeze in a prone position.

To determine whether the males and females received comparable amounts of JSS, we quantified the types and numbers of aggressive acts that each juvenile received (Table 1). There were no sex differences in the overall number of aggressive acts or in the total number of each aggressive act (*t*-tests, all p's > 0.2).

Eight juvenile females received mount(s) without intromission by an RA, and those females were removed from the RA's cage as soon as possible. A female that was mounted was not exposed to an RA again during that day, but was included in subsequent JSS episodes and all behavioral tests. We asked whether there was any relationship between the number of mounts received and any of the behavioral parameters that we measured in adulthood, and found none (all r's < 0.4 and all p's > 0.3).

2.4.2 Females more active in the open field

The number of line crosses and proportion of time spent in the inner zone of the arena per 10 min trial was measured to index locomotor activity and anxiety, respectively. Juvenile social subjugation did not affect locomotor activity within either sex (two-way ANOVA, effect of JSS: $F_{1,38} = 1.3$, $p = 0.30$), although females were more active, crossing more lines, overall than males (two-way ANOVA, effect of sex: $F_{1,38} = 5.3$, $p = 0.03$). Importantly, there was no

juvenile experience by sex interaction on locomotor activity ($p = 0.49$). Inner zone duration was not affected by sex ($F_{1,38} = 2.7$, $p = 0.10$) or by experience ($F_{1,38} = 0.01$, $p = 0.8$). Because rats rapidly adapt to the conditions of the open field, rapidly becoming less fearful over time (Blanchard and Kelley 1974) we then asked whether JSS influenced behavior during the first minute. Females were again more active ($F_{1,38} = 3.98$, $p = 0.05$). Rats subjugated as juveniles showed slightly less locomotion during the first minute than controls, but this did not reach statistical significance ($p = 0.1$). These results confirm previous studies showing greater exploratory behavior in females (Westenbroek, Ter Horst et al. 2003) and indicate that JSS does not strongly influence locomotion in the open field.

2.4.3 Sex-specific effect of JSS in the forced swim test

The Porsolt forced swim test has been widely used to assess depression-like behaviors in rodents. To determine whether JSS affected performance in the forced swim test, the rats were obliged to swim for five minutes on three consecutive days. Across all three days of testing, the duration of time spent swimming, climbing and immobile as well as the latency to become immobile was measured.

The latency to become immobile decreased among all rats over time ($p < 0.001$, Fig. 2). In addition to statistically significant main effects of sex ($F_{1,35} = 4.92$, $p = 0.03$) and experience ($F_{1,35} = 6.01$, $p = 0.02$) on immobility, there was a significant sex-by-experience interaction ($F_{1,35} = 11.50$, $p = 0.002$). Subjugated females stopped swimming significantly sooner than control females ($F_{1,35} = 17.548$, $p < 0.001$). In contrast, juvenile experience had no effect among the males ($F_{1,35} = 0.430$, $p = 0.52$). This finding indicates that adult female rats are more likely to show depression-like behavior following JSS than males.

The amount of time spent swimming and climbing during each swim trial was not affected by either sex ($F_{1,36} = 2.82$, $p = 0.102$) or experience ($F_{1,36} = 0.115$, p 0.74). Furthermore, the amount of time spent immobile on each day of swim testing was not affected by sex ($F_{1,36} = 3.127$, $p = 0.09$) or experience ($F_{1,36} = 1.86$, $p = 0.78$).

2.4.4 JSS increased closed arm time

The elevated plus maze has been widely used as an ethologically relevant measure of anxiety because it draws upon the rat's natural tendency to avoid open spaces and because anxiolytic drugs increase open arm time (Hogg 1996). We determined if JSS influenced performance in the elevated plus maze (Fig. 3). Rats that fell off the maze (n = 10 out of 40) were excluded from the analysis. There was no bias among those that fell off in terms of either sex or experience.

Subjugated rats spent a greater fraction of time in the closed arms of the maze than controls ($F_{1,29} = 8.6$, $p = 0.007$; Fig. 3A). Because we hypothesized that JSS would increase anxiety-like behavior in females more than males, we performed post hoc tests within each sex. These indicated that JSS increased closed arm time more in females ($p = 0.03$) than in males ($p = 0.07$). Consistent with this finding, JSS females had more closed arm, and fewer open arm entries (Tukey tests, all p's < 0.05). Nonetheless, it is clear from the overall pattern of the data that JSS affected males too, just to a lesser extent than females. Thus, while these data show that JSS increases anxiety-like behaviors in both sexes, the effect of JSS on elevated plus maze performance was more severe in females.

Total number of arm entries was used as a measure of generalized locomotor activity. Sex ($F_{1,29} = 2.54$, $p = 0.12$) nor juvenile experience ($F_{1,29} = 3.53$, $p = 0.07$) did not affect the total number or percentage of arm entries made by each animal. Risk assessment was measured

by counting the numbers of stretch attend behaviors made by each animal during their time on the elevated plus maze. Risk assessment behavior was not affected by either sex ($F_{1,29} = 0.83$, $p = 0.37$) nor juvenile experience ($F_{1,29} = 0.42$, $p = 0.52$).

2.4.5 Fewer investigatory approaches in subjugated females

Social withdrawal is a classic symptom of major depression and generalized anxiety (Weisberg 2009). To test whether JSS increased the apprehension toward conspecifics, we put a sex- and age-matched rat into the subject's home cage for 10 minutes. Two investigators coded the number of investigatory approaches (Fig. 4).

Although neither sex ($F_{1,35} = 2.28$, $p = 0.14$) nor experience ($F_{1,35} = 2.34$, $p = 0.14$) significantly affected the number of investigatory approaches, there was a significant interaction between sex and experience ($F_{1,35} = 4.39$, $p = 0.04$). Subjugated females apparently drove this effect because they made 27% fewer investigatory approaches than control females. In contrast, the number of investigatory approaches was indistinguishable between JSS and control males. A post hoc test confirmed this: JSS females made significantly fewer investigatory approaches than controls ($p = 0.02$), whereas males were unaffected. Changes in generalized locomotor activity did not contribute to this finding as there were no differences in the number of mid-line crossings within sex ($F_{1,35} = 2.68$, $p = 0.111$) or experience ($F_{1,35} = 0.002$, $p = 0.969$) during the social interaction test.

We also counted number of aggressive acts (pins, rear paw kicks, and dominance postures) initiated by the experimental animals in the social interaction test. Although aggressive acts were observed, neither sex ($F_{1,36} = 1.47$, $p = 0.2$) nor juvenile experience ($F_{1,36} = 2.04$, $p = 0.16$) affected their probability, nor was there an interaction between sex and juvenile experience ($F_{1,36} = 0.001$, $p = 0.9$).

2.4.6 Stress-evoked CORT levels higher in JSS females

Because HPA axis dysregulation is a classic symptom of depression, particularly in patients with a history of child abuse, we collected venous blood immediately after the first forced swim and trunk blood when the rats were decapitated in order to measure stress-evoked and basal levels of CORT. Decapitation occurred at the daily nadir of circulating CORT levels, shortly after lights out at 1900 h.

First, there was the expected sex difference in overall CORT levels, with females having higher basal ($F_{1,38} = 29.15$, $p < 0.001$), and stress evoked CORT levels ($F_{1,34} = 53.7$, $p < 0.001$) than males (Fig. 5). As expected, forced swim dramatically increased CORT levels for both males ($F_{1,37} = 128.3$, $p < 0.001$), and females ($F_{1,35} = 263.7$, $p < 0.001$). Furthermore, juvenile social subjugation was associated with higher stress-evoked CORT levels relative to baseline in both sexes, indicated by a significant interaction of time with experience ($F_{1,34} = 9.8$, $p < 0.01$). Because stress-evoked cortisol levels are higher in women that have been victimized by child abuse, we hypothesized that JSS would similarly sensitize the HPA axis in female rats, not males. *Post hoc* comparisons within the baseline and evoked conditions showed that this difference was driven by the females within the evoked condition (females, $p = 0.02$; males, $p = 0.25$). Thus, JSS sensitized the stress response in females, but not in males.

2.4.7 Heavier adrenal gland weights in JSS females

We weighed the adrenal glands, thymus gland, and seminal vesicles because prolonged stress is associated with adrenal hypertrophy (Nemeroff, Krishnan et al. 1992, Rubin, Phillips et al. 1996), thymic involution (Tarcic, Ovadia et al. 1998), and reduced male fertility (Blanchard, Spencer et al. 1995). As expected, given their higher overall CORT levels, females had heavier adrenal glands than males ($F_{1,39} = 53.2$, $p < 0.001$). Given our expectation that JSS would affect

males and females HPA axes differently, we performed post hoc comparisons within each sex. We hypothesized that JSS would increase adrenal gland weight in females more than males. Our hypothesis was confirmed: JSS increased adrenal gland weight in females ($p = 0.03$), but not in males ($p = 0.6$; Fig. 6). We repeated this analysis after normalizing adrenal gland weight by each rat's body weight, and observed an identical pattern of effects (females, $p = 0.02$; males, $p = 0.7$).

Thymus gland weights were significantly heavier in males ($F_{1,38} = 17.4$, $p < 0.001$), but juvenile experience did not affect thymus weight directly ($p = 0.3$) or interact with sex ($p = 0.5$). Once again, after body weight normalization, the same sex difference and absence of an experimental effect was observed. No effect of experience was observed on seminal vesicle weights.

2.4.8 Estrous cycle phase did not affect any dependent variable

Finally, we analyzed rats' behavioral performance by estrous cycle phase on those tests where there was a significant effect of juvenile experience. We chose to account for estrous phase *post-hoc* instead of synchronizing testing with a particular estrous phase. Synchronizing behavioral tests with an estrous phase was incompatible with our blocked testing design, which consisted of consecutive testing days and was designed to eliminate any carry-over effects of behavioral test sequence.

No effect of estrous cycle phase was seen in any day of forced swim testing (all p's > 0.4). In the elevated plus maze, however, there was an effect of phase: Proestrous females spent less time in the closed arms of the maze than estrous females ($p = 0.03$), spent more time in the open arm of the maze ($p=0.03$), made more closed arm entries ($p=0.001$) and made less open arm entries ($p=0.001$). Importantly, though, there was no relationship between juvenile experience and estrous phase for any of the above mentioned variables; the proestrous females during this

test were evenly distributed between HC (n=3) and JSS (n=4) groups. There was no effect of phase in the social interaction test ($p = 0.18$).

2.5 Discussion

The principal finding of this experiment is that 10 episodes of JSS leads to a sex-specific pattern of depression- and anxiety-like behaviors even though males and females received nearly identical numbers and types of aggressive acts: pins, rear paw kicks, and dominance postures. Because the juveniles were too small to defend themselves, these episodes did not involve any fighting; hence the use of the term juvenile social subjugation rather than "defeat". Once the animals were adults, three weeks after the conclusion of the juvenile social subjugation, performance in standard behavioral tests revealed that JSS had induced a pattern of depression- and anxiety-like behaviors that was clearly more severe in females. The mood disorder-like behaviors were (1) increased behavioral despair in the forced swim test, (2) decreased sociability in a social interaction test, and (3) increased time in the closed arms of the elevated plus maze. JSS also affected in a sex-specific manner two important physiological variables associated with mood disorders: Exaggerated CORT responses to the forced swim stressor in females, and increased adrenal gland weight in females.

These data show that JSS is a tractable, ethologically relevant manipulation that induces long-term changes in physiology and behavior. They also show that despite being given very similar adverse early experiences, males and females nonetheless were affected very differently several weeks after the experience had ended.

2.5.1 Sex-specific effects of juvenile experience: Depression-like behaviors

Consistent with the frequent co-morbidity of anxiety and depression in humans, JSS induced a behavioral phenotype marked by depression- and anxiety-like behaviors. Two aspects

of depression-like behavior were measured. First, behavioral despair was measured using the Porsolt forced swim test. The forced swim test has been widely used as an index of a depression-like state in part because of its sensitivity to anti-depressant medication such as fluoxetine (Porsolt 1979, Kirby and Lucki 1997). Furthermore, adverse experiences such as chronic variable stress (Jankord, Solomon et al. 2011), social defeat (Hollis, Wang et al. 2010), and footshock (Dunn and Swiergiel 2008) reduce swim time, suggesting that they heighten vulnerability to behavioral despair.

JSS induced the females to stop swimming sooner than their controls across all 3 days of swim testing. In contrast, there was no effect of experience among the males, indicating that JSS had induced a depression-like state only in females. The number of females in each estrous cycle phase was evenly distributed among groups and across days of the forced swim test, ruling out circulating ovarian hormones as a factor. There was an underlying sex difference in the susceptibility to behavioral despair because control males stopped swimming sooner than control females, consistent with previous reports (Barros and Ferigolo 1998, Campbell, Lin et al. 2003, Drossopoulou, Antoniou et al. 2004). Yet in spite of the sex difference in baseline latency, there was no detectable effect of JSS among the males. JSS thus had a sex-specific effect, making females more prone to behavioral despair.

Withdrawal and social phobia are common symptoms of depression and generalized anxiety disorder (Drevets, Videen et al. 1992, Sandi and Richter-Levin 2009, Davey, Allen et al. 2011). To assess rats' sociability, we measured their tendency to approach and investigate an unfamiliar conspecific introduced into the subject's home cage. Female rats that were subjugated as juveniles made fewer investigatory approaches toward a sex- and age-matched conspecific as compared to their controls. In contrast, males were unaffected by juvenile experience. As before,

ovarian hormones could not have differentially influenced behavior in this test because estrous cycle phases were evenly distributed between JSS and HC females.

2.5.2 Anxiety-like behaviors

Anxiety-like behavior was measured by time in the closed arms of the elevated plus maze. Closed arm time in the elevated plus maze has been widely used as a construct of anxiety, validated by the effectiveness of anxiolytic medication to decrease it (Hogg 1996). JSS increased closed arm time in both sexes, importantly showing that this experience could affect males. Nonetheless, the effect of JSS on this parameter was clearly greater in females: JSS females spent 23% more time in the closed arm than their controls, whereas subjugated males spent 17% more time than their controls. Although proestrous females spent more time in the closed arms of the maze, as expected from previous studies (Díaz-Véliz, Alarcón et al. 1997, Frye, Petralia et al. 2000), equal numbers of HC and JSS females were proestrous at the time of this test, again excluding ovarian hormones as a contributing factor to the effects of juvenile social subjugation.

In the open field test, females showed greater locomotion, but there was no effect of juvenile social subjugation among them or the males. Apprehension of the open field drops rapidly within the first few minutes of the test (Blanchard and Kelley 1974). Consistent with this greater fearfulness at the beginning of the test, subjugated rats made slightly fewer line-crosses during the first minute of the test, but this did not reach statistical significance.

Principal component analysis of performance in the elevated plus maze and open field indicate that they tap different dimensions of the anxiety construct, with the open field being more reflective of locomotion/exploratory behavior and the plus maze more of anxiety itself (Ramos and Mormède 1998). The open field test was originally designed to measure defecation, and later, locomotion. Only recently has it been used been as a test of anxiety-like behavior, but

this is controversial due to the confound with locomotion (Stanford 2007). The elevated plus maze may thus be more sensitive to the particular state induced by JSS. Another, non-mutually exclusive possibility is that the elevated plus maze provoked greater anxiety than the open field, and thus was more effective at revealing the effect of JSS.

2.5.3 Sex-specific effects of JSS on the HPA axis

Major depression is associated with sensitization of the HPA axis, causing exaggerated stress-evoked ACTH and cortisol levels (Heim, Newport et al. 2000, Heim and Nemeroff 2002, Heim, Newport et al. 2008). JSS apparently sensitized the HPA axis of females, leading to increased stress-evoked CORT levels. The effect of JSS was undetectable in males.

Interestingly, the adrenal glands of subjugated females were significantly heavier than those of handled controls. As one might expect, increased adrenal gland weight is also associated with depression, reflecting glucocorticoid and/or norepinephrine hypersecretion (Nemeroff, Krishnan et al. 1992, Rubin, Phillips et al. 1996). Evoked CORT levels and adrenal hypertrophy provide convergent physiological evidence that JSS dysregulated the HPA axis in a sex-specific manner. Adrenal hypertrophy suggests that CORT levels were chronically elevated in the JSS females, which is seemingly at odds with the absence of any effect of JSS in basal CORT levels. This apparent discrepancy may be due to a floor effect on basal CORT levels, measured when they were supposed to be at their lowest, during the subjective morning of the rats.

The stress response in juveniles is more protracted than in adults (Goldman, Winget et al. 1973, Vázquez and Akil 1993, Romeo and McEwen 2006), resulting in prolonged exposure to glucocorticoids, which have well-known deleterious effects on the brain. Studies of restraint stress-evoked CORT in juveniles indicate that, like adults, female rats have a greater acute response than males (Romeo, Lee et al. 2004, Romeo, Bellani et al. 2006). Although we did not

measure CORT levels after a social subjugation episode, the experiences were clearly aversive. The juveniles emitted audible cries during the subjugation, froze and fled from the RAs as the episodes progressed. Assuming that a greater stress response was mounted to JSS relative to that mounted to handling, the higher glucocorticoid levels may have affected the HPA axis differently in each sex, leading to the sex-specific effect of JSS that we observed several weeks later.

2.5.4 Similar experiences, different outcomes

Sex-specific effects of JSS on behavior and CORT levels could lead one to conclude that the rats processed their experiences in sexually dimorphic ways, perhaps because of sex differences in the brain. Yet despite receiving nearly indistinguishable levels of subjugation, experimental conditions were nonetheless different for the sexes in two ways: The females were lavaged, and some received mounts.

The experience of lavage could have interacted with JSS to exaggerate effects that might not have otherwise been sexually dimorphic. However, because lavages were initiated at P62, three weeks after the cessation of social subjugation, this seems unlikely. As noted earlier, receiving mounts had no predictive value in any of the parameters that we measured. It is also possible that differential perception of the JSS experience, by males and females, led to sex-specific mood disorder-like behaviors.

Social isolation is a significant stressor in and of itself (Lukkes, Mokin et al. 2008). It is conceivable that social experience with the RA during the social subjugation episode mitigated the effect of social isolation in males but not females, reducing any synergy between JSS and social isolation in producing mood disorder-like effects. At the same time, social isolation and JSS could have interacted to exaggerate the effect seen in females. Arguing against this scenario,

however, is the report that juvenile social isolation increases anxiety-like behavior in male, but not female, rats (Weiss, Pryce et al. 2004). In light of that finding, social isolation should have reduced sex differences, not heightened them.

The most parsimonious interpretation of our results is that JSS was responsible for the effects observed in adulthood. How then to account for the sex-specific effect of JSS? A sexually dimorphic forebrain circuit that encompasses the medial amygdala, bed nucleus of the stria terminalis, and mediobasal hypothalamus mediates the detection of social cues and the coordination of social behavior (Newman 1999, Simerly 2002). This circuit has also been identified as a key forebrain network in the control of the HPA axis, particularly for psychogenic stressors such as social defeat (Dayas, Buller et al. 1999, Dayas, Buller et al. 2001, Herman, Ostrander et al. 2005). Organizational effects of gonadal steroids alter the synaptic organization of this network such that it is sexually dimorphic before puberty (Cooke and Woolley 2005, Cooke, Stokas et al. 2007), and activational effects alter its organization still further during puberty (Cooke and Woolley 2005, Cooke 2010). We hypothesize that JSS is processed in a sexually dimorphic manner by this circuit, leading to long-term, sex-specific changes in networks that regulate mood and the stress response.

2.5.5 Conclusion

We have provided several lines of evidence that JSS influences adult behavior and neuroendocrine function in a manner that strikingly resembles the epidemiology and behavioral symptoms of mood disorders. Juvenile social subjugation was associated with sex-specific depression- and anxiety-like behaviors as well as dysregulation of the HPA axis. JSS therefore has face and construct validity as a model of stress-induced affective disorders in humans, making it a potentially useful tool to study how child abuse increases the risk of mood disorders

that are more common in women. This work underlines the importance of social experience to behavioral development and highlights adolescence as a developmental epoch of sensitivity to social stress.

2.6 Acknowledgments

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2.7 Chapter 2 Table

Table 1 Number and types of aggressive acts received by juveniles during JSS (mean ± sem)

Table 1. Total number of abusive acts received by the juveniles (mean ± sem)				
	Pins	**Rear paw kicks**	**Dominance postures**	**Total**
Male	15.2 ± 3.7	44.7 ± 10.3	113.4 ± 13.5	173.3 ± 33.9
Female	15.4 ± 5.5	67.2 ± 12.7	104.3 ± 9.3	186.9 ± 32.9

2.8 Chapter 2 Figures

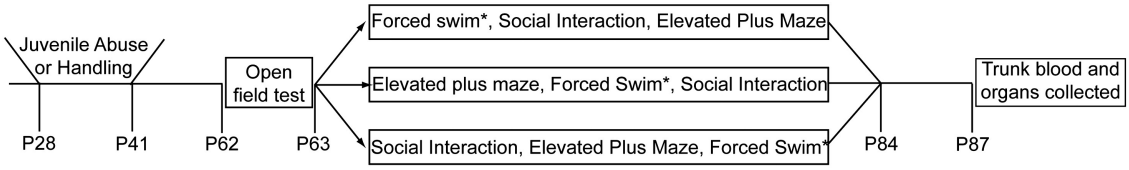

Figure 1 Experimental timeline

Juvenile rats were randomly assigned to either handling or social subjugation conditions, and had 10 of those experiences. At P62, all rats were given the open field test. Subsequently, each rat was randomly assigned to one of three groups that received three behavioral tests in counterbalanced order: Forced swim, elevated plus maze, and social interaction tests. Three days after the last behavioral test, all rats were rapidly decapitated, and trunk blood and organs were collected.

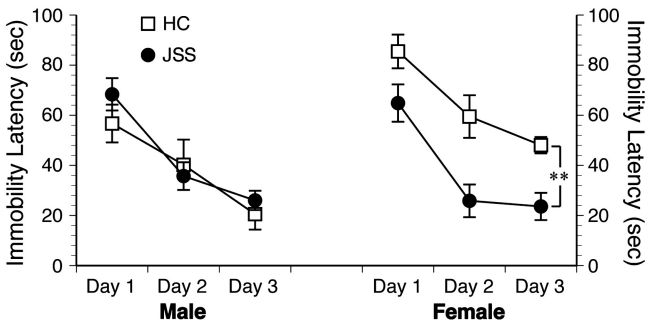

Figure 2 Juvenile social subjugation increases behavioral despair in females

The latency to stop swimming was shorter in subjugated females than in handled females, whereas juvenile social subjugation had no effect on behavioral despair in males (*Post hoc* Tukey tests, * $p < 0.05$, ** $p < 0.01$).

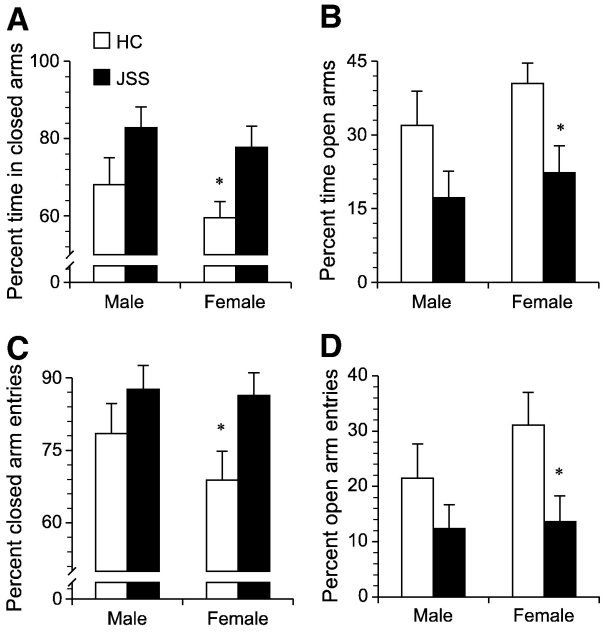

Figure 3 Juvenile social subjugation increases anxiety-like behavior in females

A - B. JSS significantly increased the fraction of time spent in the closed arms and the number of closed arm entries in females only. C. JSS significantly reduced the number of open arm entries in females only. D. HC rats tended to be more active, although this was not significant ($p = 0.07$). * *Post hoc* Tukey tests, ($p \leq 0.03$).

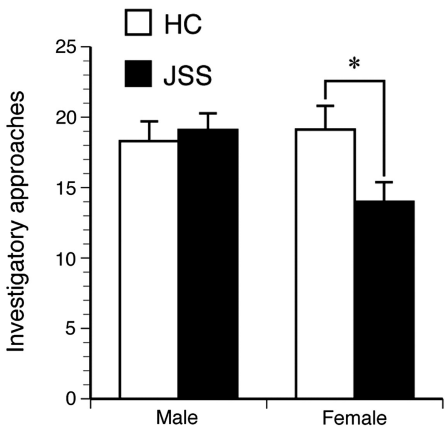

Figure 4 Juvenile social subjugation decreases sociability in females

Juvenile social subjugation increased females' apprehension of novel conspecifics (* p = 0.02).

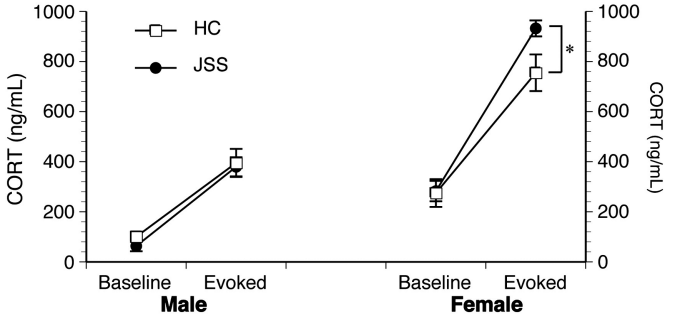

Figure 5 Sex and juvenile experience affect adult CORT levels

Points represent mean (± SEM) plasma CORT concentration measured at circadian nadir and after forced swim stress. Females had significantly higher overall CORT levels than males, and JSS significantly increased stress-evoked CORT levels in females (* p = 0.02) but not males (p = 0.25).

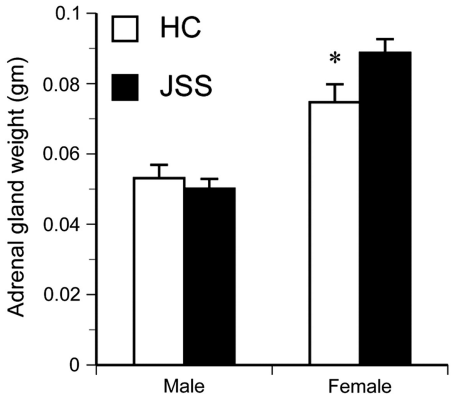

Figure 6 Sex-specific effect of JSS on adrenal gland weight

Females subjugated as juveniles had heavier adrenal glands than handled controls (*p = 0.02)

3 CHAPTER THREE: SOCIAL EXPERIENCE INDUCES SEX-SPECIFIC FOS EXPRESSION IN THE AMYGDALA OF THE JUVENILE RAT

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3.1 Abstract

To compare the response of the medial and central amygdala to juvenile social subjugation (JSS), we used unbiased stereology to quantify the immediate early gene product Fos in prepubertal rats after aggressive or benign social encounters, or handling. We estimated the overall number of neurons and the proportion of Fos immunoreactive neurons in the posterodorsal (MePD), posteroventral medial amygdala (MePV) and the central amygdala (CeA). Experience elicited Fos in a sex- and hemisphere-dependent manner in the MePD. The left MePD was selective for JSS in both sexes, but the right MePD showed a specific Fos response to JSS in males only. In the MePV, irrespective of hemisphere or sex, JSS elicited the greatest amount of Fos, benign social experience elicited an intermediate level, and handling the least. None of the experiential conditions elicited significant levels of Fos in the CeA. We found a previously unreported sex difference (M>F) in the number of CeA neurons that was highly significant, and a strong trend toward a sex difference (M>F) in the MePD. These data show that the posterior MeA subnuclei are more responsive to JSS than to benign social interaction, that sex interacts with hemispheric laterality to determine the response of the MePD to JSS, and that the MePV responds to social experience and JSS. Taken together, these findings support the hypothesis that juvenile rats process JSS in a sex-specific manner.

3.2 Introduction

Anxiety and depression are the most common stress-related mood disorders in the United States, affecting 20 million Americans annually. Teenage girls and women are 70% more likely than males to develop these complex psychiatric conditions, a sex difference that first appears during puberty (Cyranowski et al., 2000; Marcus et al., 2005; Weissman and Klerman, 1977). The disparity between the sexes, as well as the association of mood disorders with changing levels of sex steroid hormones (Becker et al., 2007; Frye and Wawrzycki, 2003; Walf and Frye, 2006), raises the possibility that sex differences in the stress response system contribute to mood disorder prevalence. The hypothalamic-pituitary-adrenal (HPA) axis is strongly modulated by perceptual and mnemonic inputs, many from brain regions that are sexually dimorphic (Herman et al., 2005). Because stress and gender are major risk factors for the development of mood disorders, we are focused on understanding how gender interacts with the stress response system to potentially increase susceptibility to mood disorders.

One of the most potent stressors for animals, including humans, is the behavior of conspecifics (Blanchard et al., 1998; Ge et al., 1994; Koolhaas et al., 1997; Young and Altemus, 2004). For example, physical abuse at the hand of an adult is a major stressor for children. Aside from sex and heredity, having been the victim of physical and/or sexual abuse as a child is the single greatest risk factor for the development of a mood disorder (Dube et al., 2001; Felitti et al., 1998; Neigh et al., 2009). Interestingly, in spite of the greater prevalence of mood disorders among women, a recent epidemiological study of child abuse and neglect indicates that boys and girls are victimized at equal rates (CWLA, 2006). Assuming that the sex disparity in mood disorder prevalence holds for those that become depressed and/or anxious as a result of child abuse, there may be sex differences in the perception and/or memory of such experiences that predisposes more women to develop a mood disorder as a result.

We have an animal model for child abuse that we term Juvenile Social Subjugation (JSS). Juvenile, prepubertal rats are given one episode of JSS or handling per day for 10 days. Each JSS episode consists of receiving aggressive acts from an aggressive adult male (the RA) over 10 minutes. In preliminary studies, we found that resident females were not as reliably aggressive as males nor did they display exactly the same types of aggressive acts. By using only resident males, the male and female juveniles received nearly identical levels of aggression and types of aggressive acts. Several weeks later, the now-adult rats are tested in a number of well-validated assays of depression- and anxiety-like behaviors. In spite of receiving nearly identical numbers and types of aggressive acts, females display more mood disorder-like behaviors (Weathington et al., 2012a). Similar results have been reported by (Bourke and Neigh, 2011), although they matched the sex of the resident aggressor to that of the juvenile. In addition, the stress-evoked level of corticosterone is significantly higher in the abused females, but not the males. While acknowledging the possibility that the gender of the RA in and of itself may contribute to the difference in mood disorder-like behaviors, these findings nonetheless suggest that the experience of JSS is processed in a sex-specific manner.

Studies that visualize the neural activity-induced immediate early gene *c-fos*, or its protein product, Fos, identified the neural circuits that process social defeat in adult rats (Martinez et al., 2002). These include brain regions that are structurally dimorphic and/or possess high concentrations of sex steroid receptors. For example, the posterodorsal medial amygdala (MePD) is reliably activated by social defeat (Boguszewski and Zagrodzka, 2005; Fekete et al., 2009; Markham and Huhman, 2008; Martinez et al., 2002; Miczek et al., 2004; Nikulina et al., 2008; Westenbroek et al., 2003), and it is sexually dimorphic in adult (Cooke et al., 1999; Hines et al., 1992; Nishizuka and Arai, 1981) and juvenile (Cooke et al., 2007a; Cooke and Woolley,

2005a) rats. The participation of the MePD in social defeat experiences suggests that its circuitry could mediate sex-specific patterns of neural activity that could conceivably lead to sex-specific outcomes later in life.

It is well established that the medial amygdala (MeA) participates in many other social behaviors besides social defeat, including social recognition (Ferguson et al., 2001), and social categorization (Samuelsen and Meredith, 2009), juvenile play (Meaney and McEwen, 1986), sexual attraction and copulation (Kelliher et al., 1999; Wersinger et al., 1993), maternal behavior (Sheehan et al., 2001), and dominant / subordinate relations (Kollack-Walker and Newman, 1995; Luiten et al., 1985; Vochteloo and Koolhaas, 1987). More recent work has begun to identify unique functional roles of its subnuclei. Studies have identified the MePV as being specialized for the detection of threats such as predator odors (Canteras, 2002; Choi et al., 2005; Dielenberg et al., 2001). Studies by (Samuelsen and Meredith, 2009) and (Maras and Petrulis, 2010a; Maras and Petrulis, 2010b) have led to the suggestion that longitudinal connections in the dorsal MeA mediate the appraisal of conspecifics for their sociobiological significance. Outputs of the MeA to the basal forebrain and hypothalamus orchestrate the behavioral, autonomic, and neuroendocrine responses to conspecifics. Because JSS is a threatening social experience, we predicted that the MePD and MePV would both be activated by the experience.

To test the hypothesis that JSS induces a sex-specific pattern of neural activity, we exposed juvenile rats to a single episode of JSS, 10 minutes with a benign adult male, or handling. Their brains were then processed to visualize the activity-induced immediate early gene product Fos in the two posterior subnuclei of the MeA and the CeA. We predicted that the pattern of JSS-induced Fos would be sex-specific in the MePD but not in the CeA, which was not known to be dimorphic. Furthermore, we hypothesized that JSS would be less effective at

activating the CeA, as it has not been implicated as often in the expression of social behavior or in responding to social stressors such as defeat.

3.3 Materials and Methods

The animal procedures used in this study were previously approved by the Georgia State University Institutional Animal Care and Use Committee and met the standards outlined in the *N.I.H. Guide for the Use of Laboratory Animals.* All rats received food and water *ad libitum*, and were housed in a reversed 12 hr light schedule (lights off at 0700 h) in a temperature-controlled ($22^{o} \pm 1$ C) animal facility.

3.3.1 Screening Aggressive Residents

Fifteen postnatal day 60 (P60) male Long Evans rats were obtained from Charles River and housed individually. Three weeks prior to the JSS procedure, these males were screened to identify those that were reliably aggressive toward juvenile rats. In the screening procedure, a P28 male or female rat was introduced into the male's home cage for ten minutes and the number of aggressive acts (pins, rear-paw kicks and dominance postures) toward the stimulus juvenile was recorded. The seven rats that made the greatest number of aggressive acts served as the RAs. To increase the aggressiveness of the RAs, cage changes were reduced to once per week and each RA was paired with an adult ovariectomized female that twice per week received subcutaneous injections of estradiol (0.1 mg) followed by progesterone (0.5 mg) 24 hours later. The RAs and their cage mates were housed in modified cages (53 x 29 x 20 cm) that allowed the insertion of a transparent perforated partition that created a compartment approximately one-third the size of the overall cage.

An additional 15 rats were screened for their docility toward juveniles. Rats that made no aggressive acts toward the juveniles were used as a "benign" resident in the experiment. From

the original cohort, 5 benign adults were selected. Benign residents were individually housed in standard laboratory cages (45 x 23 x 20 cm).

3.3.2 Juvenile Social Subjugation

Three weeks after the arrival of the RAs, 36 P21 male and female Long Evans rats were delivered from Charles River Laboratories. Subjects were housed individually in standard laboratory rat cages (45 x 23 x 20 cm) with corncob bedding. Rats were left undisturbed for one week except for feeding and cage changes. Six days after their arrival at the GSU vivarium, juveniles were randomly assigned to handled control (HC), benign control (BC) or juvenile social subjugation (JSS) groups. We then used a procedure adapted from the original resident-intruder paradigm of (Miczek, 1979). To begin the JSS procedure, the RA's female cage mate was removed, the perforated partition was inserted, and a juvenile was then introduced into the smaller compartment of the RA's cage. After five minutes, the partition was removed and the juvenile and resident were allowed to interact for no more 10 minutes or until at least 10 aggressive acts (pins, rear-paw kicks, and dominance postures) were received by the juvenile. Pins were scored when an RA immobilized the juvenile in a supine position. Rear paw kicks were scored when the RA used its rear paws to kick the intruder. Dominance postures were scored when the RA immobilized the juvenile with its front paws, causing it to freeze in a prone position. The type and number of aggressive acts was scored manually. If less than 10 aggressive acts were received by the juvenile, it was excluded from the experiment. The partition was then replaced, and the juvenile was returned to the smaller compartment for another five min, after which it was returned to its home cage, and sacrificed 60 min later. If sexual behavior was observed from the resident, that juvenile was removed from the study. None of the aggressive acts resulted in any observable injury to the juveniles. After the trial was over, female cage mate

was returned to the RA's cage. These episodes occurred between 1000 and 1300 each day during the dark cycle and were performed on juveniles between the ages of P28 and P33. The total number of JSS episodes each RA performed over the whole experiment was no more than two, and often only one.

The BC procedure resembled the JSS procedure in every way, except that the juvenile encountered a benign adult male instead of an RA for 10 min. So as to equalize sensory experiences to the fullest extent possible, the encounter with the benign male occurred in the home cage of an RA that had recently been removed. The juvenile was placed behind the perforated partition for 5 min, the juvenile directly interacted with the benign adult for 10 min, and then was replaced behind the perforated partition for another 5 min. BC juveniles were only used in the analysis if no aggressive acts were observed. As with JSS females, if any sexual behavior was observed, that BC juvenile was removed from the study. The total number of episodes each benign adult participated in was no more than two, and often only one.

HC rats were treated with identical procedures as the JSS and BC rats, including two 5 min periods behind a partition and one 10 min period exploring the cage, except that the rats were placed into a clean cage containing only fresh bedding.

Following the JSS, BC, or HC experience, each rat was housed individually in a quiet dark room for 60 min, at which point they were deeply anesthetized with pentobarbital, then perfused with 200 mL of cold 4% paraformaldehyde in 0.1M phosphate buffer. Their brains were dissected, blocked at the levels of the optic chiasm and the cerebellum, notched on the right hemisphere, and allowed to post-fix in fresh perfusate overnight. Brains were stored in a 30% sucrose solution 72 hours before serial sectioning at 40 μm in 4 series, at which point they were stored in cryoprotectant until immunohistological processing.

3.3.3 Immunohistochemistry

To visualize Fos, one series from every animal in the study was placed into multi-welled racks and washed (10 x 5 min) in 0.1 M phosphate buffered saline (PBS, pH 7.4). To quench endogenous peroxidases, the tissue was then incubated in 0.3% H_2O_2 for 15 min. Tissue sections were extensively washed in PBS, then incubated in rabbit polyclonal anti-c-Fos IgG antibody (diluted 1:10,000, Santa Cruz Biotechnology, SC-7202) for 48 hrs at 4°C in PBS containing 0.4% Triton X-100. Sections were washed, incubated in biotin-SP-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:600, Jackson Immunoresearch Inc) for 1hr at RT, then washed again in PBS. Sections were then incubated in an avidin-biotin solution (ABC peroxidase 'Elite' kit, Vector Laboratories) in PBS containing 0.4% Triton X-100 for one hour at room temperature. Sections were washed in PBS, then in a solution of 0.175 M sodium acetate. The tissue was incubated in 0.175 M sodium acetate solution containing 3% H_2O_2, 2.5% nickel ammonium sulfate, and 5% w/v 3, 3, diaminobenzidine (Sigma, cat. # 32750) for 15 minutes at RT. Lastly, sections were washed, returned to 0.1 M PBS, and stored in 0.1 M phosphate buffer at 4 °C until mounting onto coded slides.

Tissue sections were mounted in serial order on gelatin-coated slides and dried overnight at 37 °C. Sections were counterstained with cresyl violet, dehydrated in a series of ascending concentrations of alcohols, cleared in xylene, and coverslipped with Permount.

3.3.4 Stereology

We used the stereology to provide an unbiased estimate of the total number of Fos+ and Fos- neurons. To account for potential sex differences in the overall number of neurons, we estimated the total number of neurons in order to calculate the proportion of Fos+ neurons. Although the absolute number of Fos+ neurons may be biologically meaningful, its significance

is ambiguous if one sex has more neurons overall. Thus, we report the overall number of neurons and the proportion of Fos+ neurons in each brain nucleus, even if that area did not have sex differences.

Unbiased estimates of overall neuron number and the number of Fos+ neurons were conducted using Stereo Investigator (MBF Biosciences, Williston, VT, USA) under brightfield illumination using a Zeiss M2 AxioImager microscope (Carl Zeiss, Okerkochen, Germany). An assistant coded the slides to ensure that group membership was unknown during analysis. To begin the analysis, each brain region was traced at 2.5x magnification using the (Swanson, 1992) rat atlas as guides. Each hemisphere was traced and counted separately. To count neurons, the investigator used a Plan Apochromat 100x oil-immersion objective (1.4 NA) and a systematic-random sampling scheme to classify all neurons that fell within the inclusion lines of the counting frame as Fos+ or Fos-. A Fos+ neuron was identified if it contained a black DAB precipitate in its nucleus; Fos- neurons had a clear nucleus, and a lightly stained cytoplasm (Fig 1A, inset). Neurons were distinguished from glia by their greater size and lighter staining. Fos labeling was not observed in glial cells. The counting frame size was 25 μm^2 for all regions of interest whereas the x- and y-dimensions of the sampling grid varied for each region. Variable grid size was necessary to ensure that 150 – 200 neurons were counted for every brain area per hemisphere, resulting in a coefficient of error (CE) of < 0.1 for each brain region in each hemisphere. Counting frame height was 6 μm with 2 μm guard zones. The mean section thickness was 12 μm. The optical fractionator estimates the number of neurons N as follows:

$$N = \left(\sum N\right)\left(\frac{1}{ssf}\right)\left(\frac{1}{asf}\right)\left(\frac{1}{tsf}\right) N = \left(\sum N\right)\left(\frac{1}{ssf}\right)\left(\frac{1}{asf}\right)\left(\frac{1}{tsf}\right)$$

$\sum N$ is the sum of neurons counted, the section sampling fraction (ssf) is the number of sampled sections divided by the total number of sections through the brain area, the area sampling fraction (asf) is the total area sampled by the counting frames divided by the total area of all sampled sections, and the thickness sampling fraction (tsf) is the height of the disector divided by the average section thickness.

Maps of Fos+ neurons

To create maps of Fos+ neurons, we exported the coordinates of each contour and marked Fos+ neuron from StereoInvestigator and overlaid each contour plus its labeled cells on its corresponding rat brain atlas plate (Swanson, 1992) in Adobe Illustrator. Tracing size was adjusted to match the atlas, whereupon marks corresponding to Fos+ neurons were superimposed on a separate layer. These marks were color coded and equal in diameter to the sampling grid size for that brain region.

3.3.5 Statistics

We first analyzed the proportion of Fos+ neurons with a two-way repeated measures analysis of variance (ANOVA), with cerebral hemisphere as the within-subjects variable, and sex and experience as the between-subject variables. If laterality was not detected, we collapsed the data in each group across the hemispheres, and analyzed the proportion of Fos+ neurons with two-way ANOVAs to identify sex differences, effects of experience, or sex differences in the response to experience. Statistical significance among any of these factors led us to analyze the Fos data within each sex using one-way ANOVAs followed by Tukey HSD *post hoc* tests. If an effect of laterality was detected, we analyzed each hemisphere separately with two-way ANOVAs followed by Tukey HSD post hoc tests. All pair-wise comparisons were performed using student's t-tests.

We then determined whether the proportion of Fos+ neurons varied as a function of rostro-caudal position within the brain nucleus as follows: The number of Fos+ markers was divided by the total number of markers in each traced contour. This was necessary to account for the variable number of neurons available to be counted in each contour. The data from each contour were assigned to a bin corresponding to the plate in Swanson (1992) that most closely matched the drawn contour. The data were then arcsine transformed and analyzed with a repeated measures two-way ANOVA. Alpha was set equal to $p < 0.05$.

3.4 Results

3.4.1 Behavior

We begin our description of these results by summarizing the encounters between the juveniles and the RAs. As mentioned earlier, rats were included in the study only if they received at least 10 dominance postures, rear-paw kicks, and/or pins in ≤ 10 min. This standard ensured that males and females received an equal number of aggressive acts (male vs. female *t*-test, $p = 1.0$). Furthermore, there were no sex differences in the number of each type of aggressive act (*t*-tests, p's range from 0.51 – 0.92). On average, each juvenile male received 2 ± 0.8 pins, 3 ± 1 rear-paw kicks, and 6 ± 1 dominance postures and each juvenile female received 2 ± 1 pins, 4 ± 1 rear paw kicks, and 5 ± 1 dominance postures during the JSS episode. In contrast, none of the encounters with the benign adult males involved any visible aggression. Indeed, the encounters with the benign adult included extensive prosocial contact, such as sniffing, playing, and side-by-side positions. Sixty minutes following the conclusion of the JSS and control episodes, the rats were perfused to visualize Fos protein.

3.4.2 Neuroanatomy

3.4.2.1 The posterodorsal medial amygdala

The MePD was traced according to the outlines in Swanson (1992; Fig. 1 A). Estimates of the overall number of MePD neurons showed that males have, on average, 46,917 neurons whereas females have an average of 39,711 neurons in each hemisphere. Although the magnitude of this effect was large (Cohen's $d = 5.17$), it was not statistically significant ($F_{1,66}=3.98$, $p = 0.0503$, Fig. 1 B).

Experience influenced the proportion of Fos+ neurons ($F_{2, 29}=5.02$, $p = 0.01$) and the rats' sex interacted with cerebral hemisphere to influence the experience-driven Fos response ($F_{2, 29} = 3.62$, $p = 0.03$). This experience-by-sex-by-hemisphere interaction was due to the robust response of both sexes in the left hemisphere (Fig. 1 C) as compared to the right hemisphere (Fig. 1 D), where males responded to JSS and females did not.

Within the left hemisphere, there was a main effect of experience on Fos expression ($F_{2,29} = 7.3$, $p = 0.002$), with no effect of sex ($p = 0.74$). Tukey tests revealed that JSS activated the MePD to a significantly greater extent than handling ($p = 0.005$), and that JSS activated the MePD to a significantly greater extent than benign social experience ($p = 0.004$).

Although JSS apparently induced Fos in the right hemisphere of males but not females, the interaction term of sex with condition was not statistically significant ($p = 0.12$). To evaluate this in another way, we analyzed right hemisphere sample means within each sex using a one-way ANOVA. This confirmed our impression, as it showed that while experience differentially affected neural activation in males ($F_{2,14} = 4.32$, $p = 0.03$), it was utterly ineffective in the right hemisphere of females ($p = 0.99$).

We mapped the position of every counted Fos+ MePD neuron in three males and females representative of each group's mean onto standard atlas plates (Fig. 2). Confirming the previous ANOVA, these showed a progressive increase in Fos+ neurons across the three conditions. There were no apparent 'hot spots' of activity, no apparent effect of medio-lateral position, or any interaction of these dimensions with sex or condition. The repeated measures ANOVA showed an effect of rostro-caudal level ($F_{2,58} = 10.10$, $p < 0.001$) because the proportion of Fos+ neurons decreased from rostral to caudal across the three conditions. There were no interactions with experience or sex on the proportion of Fos+ neurons in the MePD (all p's ≥ 0.35).

3.4.2.2 The posteroventral medial amygdala

The MePV was traced according to the outlines in Swanson (1992; Fig. 1 A). Estimates of the overall number of neurons in the MePV indicated no sex difference ($p = 0.98$) or laterality ($p = 0.24$; Fig. 1 E). Males have on average 36,941 neurons and females have 36,715 in each hemisphere.

Since hemispheric laterality did not interact with sex or condition, the data were pooled across hemisphere and analyzed with a two-way ANOVA. This revealed that experience differentially affected the proportion of Fos+ cells ($F_{2, 35} = 8.28$, $p = 0.001$) with no effect of sex ($p = 0.65$; Fig. 1 F). The data were thus analyzed with *post hoc* Tukey tests. These tests showed that JSS induced significantly more Fos than benign social experience ($p = 0.03$), which induced significantly more Fos than handling ($p < 0.001$).

We mapped the position of the Fos+ cells onto a standard atlas (Fig. 2) from three representative males and females in each group. This highlighted the responsiveness of the MePV to JSS; in both sexes, Fos was expressed throughout the MePV irrespective of rostro-caudal level and with no apparent hot spots. This impression was supported by the result of the

repeated measures ANOVA, which did not detect any effects of rostro-caudal level, or interactions with sex or experience (all p's ≥ 0.245).

3.4.2.3 The central amygdala

The CeA was traced according to the outlines in Swanson (1998; Fig. 3 A). Estimates of the overall number of CeA neurons revealed that males have on average, 116,131 neurons in each hemisphere, whereas females have 93,690 neurons, a sex difference of approximately 22,000 neurons ($F_{1,62}$=10.31, p = 0.009, Fig. 3 A), with no effect of hemispheric laterality (p = 0.99).

The proportion of Fos+ neurons in the CeA was quite low compared to the MeA. On average, only 2% of the neurons were activated in any of the conditions. There was no effect of laterality (p = 0.772) or interactions with laterality (p's ≥ 0.27). The data from each hemisphere were thus combined and analyzed. No main effects of sex or experience or interactions were detected in the proportion of Fos+ neurons (all p's ≥ 0.30; Fig. 3 B).

3.5 Discussion

In this study, we have shown that the MeA and CeA respond very differently to social experiences. As a whole, the posterior MeA was far more responsive to JSS than the CeA. However, the posterodorsal and posteroventral MeA responded in distinct ways to the social stimuli; the MePD response was sex-specific and lateralized, whereas the MePV was not. Overall, the results show that the MePD responds in a sex-specific manner to JSS, while the MePV and CeA do not. We also found a near-significant sex difference in MePD neuron number, and a highly significant sex difference in the CeA.

Our findings in the MePD were interesting for several reasons. Because of its association with many social behaviors, we expected the MePD to show a pattern of activity similar to that actually observed in the MePV, wherein both benign and aggressive social experiences induced Fos expression. Instead, the MePD was activated solely by JSS. If the MePD appraises sensory cues for biological significance, what about JSS in particular distinguished it from BC? The olfactory environments encountered by the JSS and BC juveniles were slightly different: The JSS juvenile was placed into a cage where the odors from the soiled bedding and the resident were congruent, whereas the BC juvenile encountered a male whose odor cues were different from those emanating from the bedding. The amount of time in close proximity to the adult male was not measured, but the intensity of odor cues from the benign adult was probably somewhat greater, since the JSS juvenile did not seek out the company of the adult as the BC juvenile did. Unless the MePD responds selectively to the incongruency between bedding odor and conspecific odor, it seems unlikely that differences in the olfactory environment could explain its selectivity for JSS. Instead, the MePD may have classified the behavior of the adult males as either aggressive or benign. Behavior in this case was the aggressive acts by the RA and the prosocial acts by the benign adult. The juvenile also most likely heard the males make context-specific ultrasonic vocalizations. The representation of these acts in the MeA was presumably encoded in the activity of noradrenergic, monoaminergic, and thalamic afferents, as well as in cortical areas such as the subiculum, insula, and infralimbic prefrontal cortex that project to the MeA (McDonald et al., 1999; Ottersen, 1980, 1982; Ottersen and Ben-Ari, 1979).

Statistical comparison of Fos expression along the rostro-caudal axis, and visual inspection of the map indicates that the difference between benign social experience and JSS in the MePD is quantitative. Being aggressed simply activated a larger fraction of MePD neurons

than social interaction. This finding contrasts with those in studies that compared Fos expression after different phases of male sexual behavior or compared Fos expression after sexual behavior vs. aggression. These revealed distinct patterns in the MeA, suggesting the presence of microcircuits that mediate each behavior (Coolen et al., 1997; Kollack-Walker and Newman, 1995; Veening et al., 2005). The quantitative rather than qualitative differences in Fos expression suggests that BC and JSS do not activate distinct microcircuits. Rather, the difference in Fos expression may have been due primarily to the greater intensity of JSS-related sensory stimuli, and secondarily to the distinct interoceptive feedback from the activated autonomic nervous system and stress response systems.

We previously reported a synaptic sex difference in the left MePD (Cooke et al., 2007b; Cooke and Woolley, 2005a). Sex differences at the ultrastructural level have been reported by others as well (Nishizuka and Arai, 1981). Given that there is a lateralized synaptic sex difference, one might have expected the left MePD to demonstrate sex-specific activity. Instead, the opposite pattern was observed: JSS evoked activity in the right MePD of males not females. Furthermore, since JSS evoked neural activity in the MePD is sex-specific and lateralized, what could be the functional relevance of the sex difference in neuron number, which is not lateralized? It is remarkable that two sex differences in a brain structure were not closely paralleled by a sex difference in function. It may be that these particular sex differences have no functional relevance in this behavioral context. Or, in the case of the synaptic sex difference, its function may instead be to equalize what would otherwise be a sex-specific effect, as (De Vries and Panzica, 2006) have suggested is the case with the sexually dimorphic vasopressin system.

The response of the MePV was straightforward relative to the MePD. Equally in males and females and across the hemispheres, benign social experience and JSS induced distinct levels

of neural activity, each one significantly greater than the other and from handling. If the MePD required an experience with the intensity of JSS to be activated, the MePV must then be more sensitive to social experiences overall. Alternatively, if the MePV is truly selective for threatening stimuli, then it may be that, in spite of its overtly prosocial behavior, the benign adult poses a risk that is detected by the MePV. This could perhaps be related to the adult male's greater size.

The MePD and MePV make topographic projections to targets in the basal forebrain and hypothalamus and share intense reciprocal connections with each other (Canteras et al., 1995). The MePV exchanges reciprocal connections with several areas whose projections avoid the MePD and support memory, motivation, the coordination of the stress response and overt defensive behaviors (Canteras, 2002; Choi et al., 2005; McDonald et al., 1999; Ottersen, 1980). The distinct responses of the MePD and MePV to JSS lead one to predict that the unique targets of these areas will demonstrate similar patterns of activity. However, these downstream areas also exchange reciprocal connections with each other that are postulated to 'gate' the expression of reproductive vs. defensive behaviors according to context (Canteras, 2002). The answer to why the MePV responded in a distinct manner from the MePD will ultimately come from a greater understanding of the microcircuitry of the MeA itself and how the MeA functions as part of a network that encompasses the amygdaloid complex, sensory areas, the bed nucleus, and the hypothalamus.

Compared with the MeA, the CeA demonstrated very low Fos expression in response to any of the conditions. This could indicate that the experiences were too mild to activate the CeA. It has been proposed that the brain categorizes stressors by type and utilizes distinct neural circuits to process them (Li et al., 1996). Physical stressors are reportedly more effective at

activating the CeA, whereas psychogenic stressors are more effective at activating the MeA (Dayas et al., 2001). Our results are consistent with that view. (Martinez et al., 1998) reported that a 10 min social defeat episode evoked CeA *c-fos* mRNA in adult male rats. The discrepancy between this result and ours could be due to non-mutually exclusive factors such as the intensity of the experience, and the presence of sex steroid hormones in the adult male rats. At P28-P33, rats have not yet initiated puberty (Harris and Levine, 2003; Sisk et al., 2001) and have nearly undetectable levels of androgens and estrogen (Cooke and Woolley, 2005a). Because the adult CeA expresses moderate levels of sex steroid hormone receptors (Shughrue et al., 1997; Simerly et al., 1990), gonadal hormones acting during puberty and into adulthood could potentiate the CeA in such a way as to increase the salience of social subjugation such that it can stimulate neural activity.

There was a sex difference in the overall number of CeA neurons, with males having approximately 8% more than females. To our knowledge, this is the first report of a sex difference in CeA cell number. Relatively weak by comparison to the MeA, the developing CeA nonetheless expresses detectable levels of androgen receptor and estrogen receptors mRNAs (McAbee and DonCarlos, 1998; Osterlund et al., 1998), making it in principle capable of sexually differentiating in response to the neonatal surge of androgen.

In a previous report (Weathington et al., 2012a), we found that 10 episodes of JSS over 10 days induced depression-like behaviors and hormonal responses that were more severe in females. Of course, we cannot anticipate whether nine more episodes of JSS would diminish, magnify, or leave unchanged the Fos response. We do know, however, that there was a sex-specific neural response after a single episode, demonstrating that males and females do process

these experiences differently. It will be interesting to compare neural activity after chronic JSS with the acute effects reported here.

The conclusions that could have been drawn from (Weathington et al., 2012a) were weakened by the absence of a social control, i.e., one in which juveniles are exposed to an adult male rat that does not show any aggression whatsoever. Because JSS preferentially activated the left MePD and was more effective at activating the MePV than BC, these findings show that juvenile rats can classify aggressive behavior independently of the sex of the actor, and support the notion that the experience of JSS itself contributed to the behaviors that we reported in the earlier paper.

It is interesting, though, that the right MePD of females failed to distinguish between the three conditions, while it responded selectively to JSS in males. When considered in the context of our earlier report, this could be taken to mean that females are more likely to perceive BC males to be as threatening as JSS males and/or that the greater selectivity for JSS in males may somehow protect them from the deleterious effects of JSS later in life. Recent findings suggest that in humans too, emotionally arousing images and social stimuli are processed in a sex-specific and lateralized manner within the amygdala (Armony and Sergerie, 2007; Cahill et al., 2004). These functional imaging data parallel our results quite closely in that males and females processed stimuli differently in each hemisphere of the amygdala. Taken together with the convergent data we present here, hemispheric laterality in the processing of social stressors may reflect an underlying mechanism that predisposes men and women to perceive and remember the same social stressor in a sex-specific way.

3.6 Acknowledgements

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3.7 Chapter 3 Figures

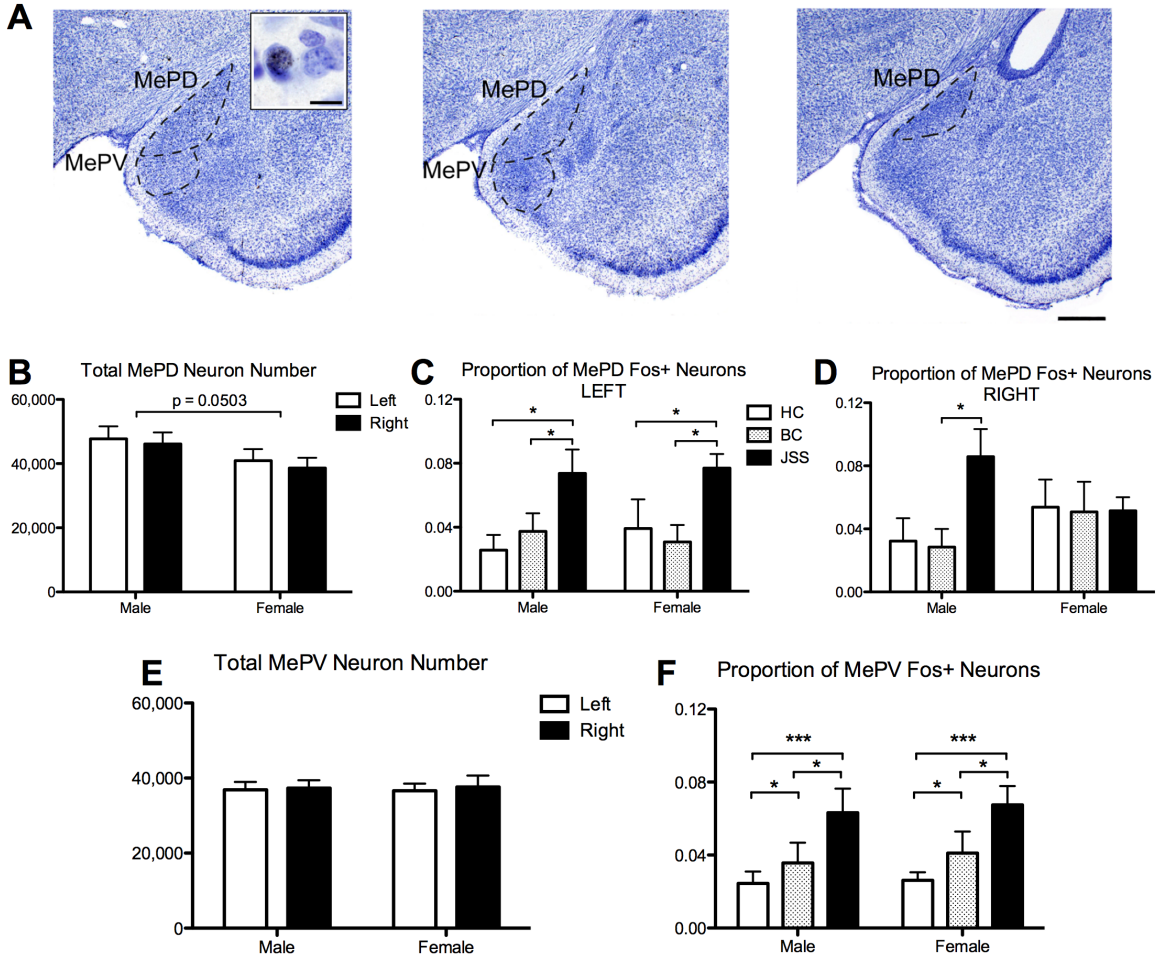

Figure 7 Fos expression in the MeA following juvenile social subjugation, benign control, or handling experience

A. Photomicrographs of the MeA at three rostro-caudal levels. Bar = 500 µm. **Inset:** Fos+ and Fos- neurons. Bar = 10 µm. **B**. The total number of neurons was not significantly greater in the male MePD. **C.** The proportion of Fos+ neurons in the left MePD. The left MePD was selective

for JSS in both males and females. **D.** The proportion of Fos+ neurons in the right MePD. JSS activated the right MePD only in males. **E.** No sex difference or laterality in the overall number of MePV neurons. **F.** The proportion of Fos+ neurons in the male and female MePV, presented separately for clarity. Experience influenced Fos expression equally in males and females; benign social experience was greater than handling, JSS was greater than benign social experience. Tukey HSD tests, * $p < 0.05$; *** $p < 0.001$.

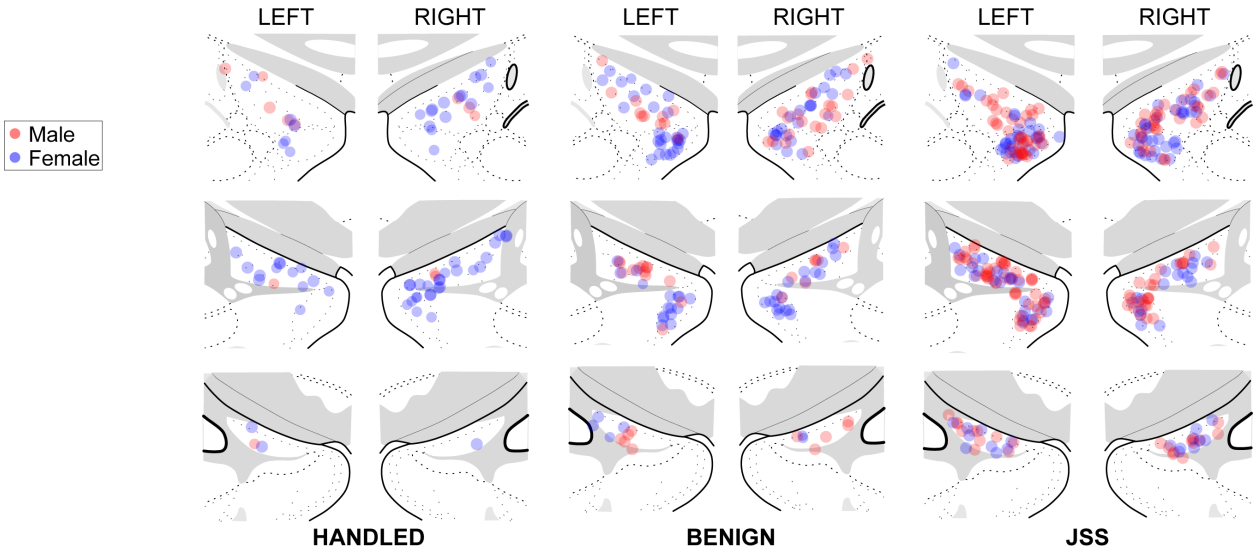

Figure 8 Map showing the rostro-caudal distribution of Fos+ markers from three representative males and females in each group within the MePD and MePV.

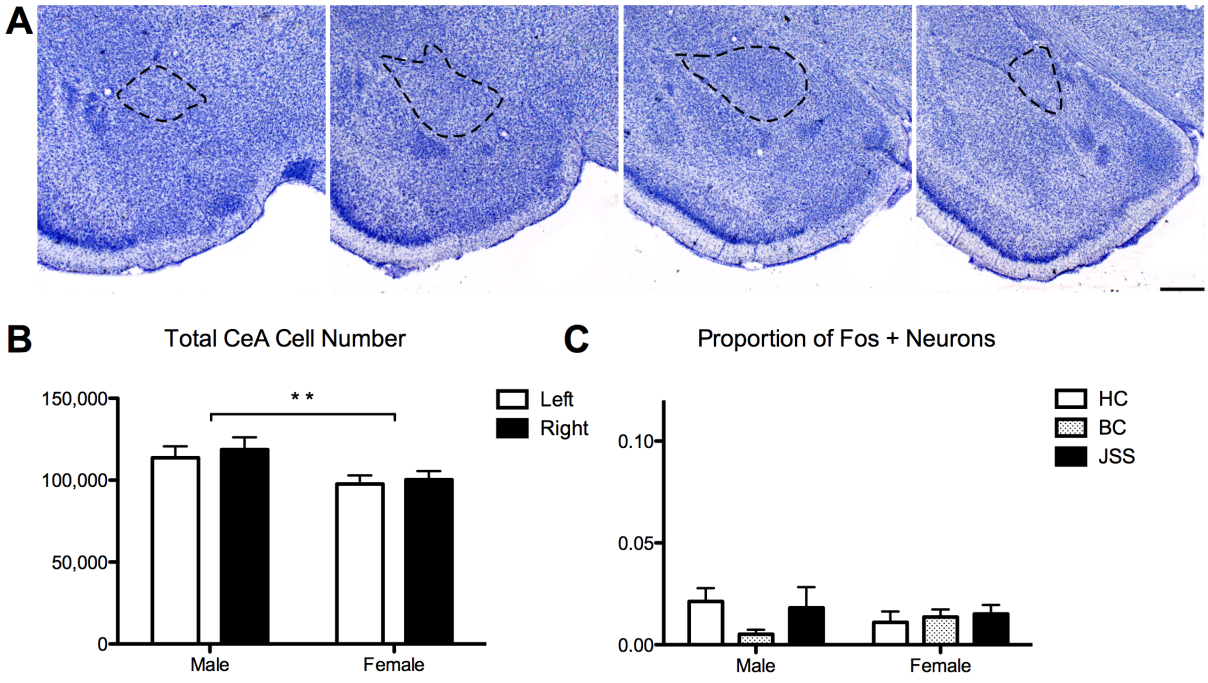

Figure 9 Fos expression in the CeA following juvenile social subjugation, benign control, or handling experience

A. Photomicrographs of the CeA at four rostro-caudal levels. Bar = 100 µm. **B**. The overall number of CeA neurons was greater in males, irrespective of hemisphere (** p = 0.009). **C**. The proportion of Fos+ neurons was unaffected by experience, by sex, or by an interaction between factors.

4 CHAPTER FOUR: SEXUALLY DIMORPHIC PATERNS OF NEURAL ACTIVITY IN RESPONSE TO JUVENILE SOCIAL SUBJUGATION

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4.1 Abstract

After experiencing juvenile social subjugation (JSS), adult female rats display more severe depression- and anxiety-like behaviours than adult males, suggesting that JSS is encoded in a sex-specific manner. To test this hypothesis, prepubertal rats (P28 – 33) were subjected to 10 aggressive acts in ≤ 10 min from an aggressive adult male, a 10 min encounter with a non-aggressive adult male, or 10 min in an empty, clean cage (handling). We then used unbiased stereology to estimate the total number and proportion of neurons immunoreactive for the immediate early gene product Fos bilaterally in the basolateral amygdala (BLA), the anterior and posterior subdivisions of the bed nucleus of the stria terminalis, and the paraventricular nucleus of the hypothalamus (PVN). Overall, females' Fos responses were less selective than males'. The BLA in males displayed a selective Fos response to the non-aggressive male, whereas no such selectivity occurred in the BLA of females. Additionally, there were more neurons overall in the left BLA than the right and this lateralization was specific to males. The principal subdivision of the BST (BSTpr) in males responded selectively to JSS, whereas the BSTpr in females was selective for both the non-aggressive and aggressive males. We also found that the regional volume and neuron number of the BSTpr is greater in males than in females. Finally, the PVN in

males was, like the BLA, selective for the non-aggressive male, whereas none of the experiences elicited a selective response in females. The greater selectivity for non-threatening stimuli in males in three stress-responsive brain regions may be a clue as to why males are less susceptible to the anxiogenic effects of JSS.

4.2 Introduction

The prevalence of stress-related mood disorders in women is twice that of men (Kessler, 2003; Tolin and Foa, 2006). The underlying diathesis that controls women's susceptibility to psychiatric conditions such as anxiety and depression is not well understood. Nonetheless, the sex difference in mood disorder prevalence suggests that circulating gonadal hormones alter brain circuits involved in mood and emotion in such a way as to protect males and/or make females more vulnerable to stress. Indeed, before puberty, mood disorders are rare and boys and girls suffer from them at roughly equal rates. The achievement of puberty, however, coincides with an increasing tendency of more women to develop mood disorders (Nolen-Hoeksema and Girgus, 1994).

In addition to the important role of gonadal hormones during puberty on mood disorder development, epidemiological data indicate that men and women are differentially susceptible to adverse early experiences encountered before puberty. Child abuse is the most potent adverse early experience in terms of the risk of developing a mood disorder, particularly one with co-morbid dysregulation of the stress response system (De Bellis et al., 1999b; Kendler et al., 1999; Tarullo and Gunnar, 2006; Widom et al., 2007). When factors such as the severity, frequency, and age of abuse are controlled for, the association between child abuse and later mood disorder development is much stronger in women than in men (Bremner et al., 2003; Cicchetti and Rogosch, 2002; MacMillan et al., 2001; Rind et al., 1998; Tarullo and Gunnar, 2006; Vigod and

Stewart, 2009). Indeed, one report indicates that in spite of the men in their sample receiving higher levels of child abuse than the women in their sample, the men were less likely to develop anxiety and/or depression than the women (McClellan et al., 1997).

We use an animal model to explore the interaction of gender with adverse early experience (Weathington et al., 2012a). In our model, prepubertal male and female rats are exposed to aggressive acts from an adult male. Each episode of juvenile social subjugation (JSS) consists of 10 minutes of direct contact with the adult, and two 5-minute episodes of non-contact exposure through a clear, perforated partition prior to and following the direct interaction. Over the course of 10 episodes of JSS, males and females receive very similar numbers and types of aggressive acts. Yet, in spite of having similar experiences, JSS affects adult females more severely than males. Specifically, JSS females are more likely show depression- and anxiety-like behaviours, including greater immobility in the forced swim test, more time in the closed arms of the elevated plus maze, more social aversion, and higher stress-evoked corticosterone than their handled controls or JSS males. This effect is independent of estrus cycle phase. A result consistent with our findings was reported by Bourke and Neigh (2011), although this group used sex-matched adults to subjugate the juveniles.

These observations prompted us to look for the neural basis of this sexually dimorphic reaction to adverse experience, and to focus on the acute immediate early gene response to a single episode of JSS. One way that females could be more adversely affected by JSS is that they encode this experience with more intense neural activity or in different subcortical microcircuits. In a previous study, we used the immediate early gene product Fos to map and compare the responses of the central and medial nuclei of the amygdala to a single, well-controlled episode of JSS, handling, or exposure to a non-aggressive, “benign” adult male rat (Weathington et al.,

2012b). To identify functional sex differences, we used unbiased, random-systematic sampling to count Fos+ and Fos- neurons. The central nucleus showed virtually no response to JSS or the control conditions whereas the Fos response in the posterodorsal medial amygdala (MePD) was more than four-fold greater. Most importantly, the MePD had a lateralized, sex-specific response: Males showed bilateral activation that was selective for JSS, whereas females showed a JSS-selective response only in the left MePD. The response in the right MePD of females was elevated for every stimulus and not selective for JSS.

Prima facie, these results did not support our hypothesis that females have a more intense response to a single episode of JSS than males. Instead, the female MePD seems less capable of distinguishing between the three distinct stimuli. However, the medial amygdala is one node in a highly interconnected network that coordinates the stress response and includes the BST and the PVN, among other regions (Herman et al., 2005). Perhaps such sites downstream of the MePD demonstrate more intense or selective responses to JSS in females than in males. A non-mutually exclusive possibility is that sex-dependent functional lateralization may contribute to sex-specific responses to JSS in adulthood. A third possibility is that repeated episodes of JSS are necessary to reveal sex differences in the magnitude and/or pattern of Fos expression.

To explore the first two alternatives, we have extended our analysis of JSS-induced neural activity to the basolateral amygdala (BLA), the BST, and the PVN – all of which are known to be involved in the appraisal of fearful stimuli, the regulation of anxiety and fear, and the coordination of behavioural and neuroendocrine stress responses (Choi et al., 2008; Lebow et al., 2012; Sigurdsson et al., 2007; Walker et al., 2009). In each of these areas, we employed the same approach as described earlier: Using the optical fractionator, we calculated the proportion of Fos+ neurons elicited in response to JSS and BC relative to a handled control (HC).

4.3 Materials and Methods

Animals

The Georgia State University Animal Care and Use Committee approved the methods used in this study. Long-Evans rats were obtained from Charles River and provided with freely available food and water. They were housed in standard shoebox-style cages in a reversed 12:12 light/dark cycle (lights off at 0700) at 22 ± 1ºC.

4.3.1 Screening aggressive residents

Fifteen postnatal day (P) 60 male rats were obtained and housed individually. Three weeks prior to the experiment, these rats were screened to identify the most reliably aggressive using the following procedure: A stimulus juvenile (P28) male or female rat was placed in the adult male's cage, and the number of aggressive acts (kicks, pins, and dominance postures) was recorded for 10 minutes. The seven most aggressive males were subsequently used in the JSS condition and were termed Resident Aggressors (RAs). To increase the territoriality of the RAs, cage changes were reduced to once a week and an ovariectomized female was housed with the RA. This female was periodically injected with estradiol benzoate (0.1 mg) and progesterone (0.5 mg) to induce behavioural estrus. The RAs and their female cage mates were housed in modified shoebox-style cages (53 x 29 x 20 cm) that permitted the insertion of a clear, perforated, plastic partition, which created a compartment roughly 1/3 the size of the overall cage.

A similar procedure was adopted to identify the non-aggressive BC animals from an additional 15 adult male rats. Only adult males that showed no overt aggression qualified as a BC.

4.3.2 Juvenile social subjugation

Male and female juvenile rats (P21) arrived at the facility and were housed individually for six days. They then were randomly assigned to one of three groups: Handling (HC), BC, or JSS. The JSS procedure occurred as follows: Between 1000 and 1300 hrs, the female was removed from the RA's cage and the juvenile was placed behind the perforated partition for 5 min. The partition was then removed and the RA and juvenile were allowed to interact for no more than 10 min or until 10 aggressive acts had occurred. If less than 10 aggressive acts occurred in 10 min or if sexual behaviour took place, that juvenile was excluded from the study. Aggressive acts consisted of rear-paw-kicks, pins (the juvenile lays in a supine position underneath the RA), and dominance postures (the juvenile freezes in a prone position underneath the RA).

Because an adult male is much heavier than a juvenile and far more aggressive, JSS consists of the unreciprocated aggressive acts of the RA. These episodes are therefore less intense than is seen in fights between adult rats and never included biting, lateral attacks, or other postures normally seen in adult fighting.

BC rats were treated identically to the JSS rats, except that their encounter was with a non-aggressive adult male in the home cage of an RA that had recently been removed. If a single aggressive act or sexual behaviour was observed, the juvenile was withdrawn from the study. After the physical encounter with the RA or BC adult male, the juvenile was returned to behind the partition for another 5 min.

The HC were treated identically to the BC and JSS rats, except that they were placed into a clean, empty cage containing fresh bedding. After these procedures, each rat was returned to its home cage and perfused 1 hr later.

4.3.3 Tissue processing

After deeply anesthetizing each rat, it was transcardially perfused with 200 mL cold 4% paraformaldehyde in phosphate buffer (PB). The brain was extracted, blocked, notched on the right hemisphere, and post-fixed overnight in fresh paraformaldehyde. Brains were then submerged in 30% sucrose in advance of serial sectioning. Brains were cut coronally (40 μm) on a freezing microtome into 4 parallel series. They were then stored in cryoprotectant at -20 ºC until immunohistochemistry was performed to visualize Fos.

To visualize Fos, freely floating sections were washed first with PBS, then with 0.3% H2O2 to remove endogenous peroxidases, washed again, and then exposed to rabbit polyclonal anti-c-Fos IgG antibody (1:10,000, Santa Cruz, SC-7202) for 48 h at 4° C in PBS with 0.4% Triton X-100. Following the primary antibody incubation, sections were washed, incubated in biotin-SP-conjugated goat anti-rabbit IgG (1:600, Jackson) for 1 hr at room temperature, and washed again in PBS. Sections were then incubated in an avidin–biotin solution (ABC peroxidase 'Elite' kit, Vector Laboratories) in PBS containing 0.4% Triton X-100 for 1 hr at room temperature. After washing, sections were placed in 0.175 M sodium acetate, followed by incubation in a 0.175 M sodium acetate solution containing 3% H2O2, 2.5% nickel ammonium sulfate, and 5% w/v 3, 3, diaminobenzidine (Sigma, cat. # 32750) for 15 min. Lastly, sections were washed, returned to 0.1 M PBS, and stored in 0.1 M phosphate buffer at 4 °C until mounting in serial order onto gelatin-coated, coded slides. They were dried overnight at 37 °C, counterstained with cresyl violet, dehydrated in a series of ascending concentrations of alcohols, cleared in xylene, and coverslipped with Permount.

4.3.4 Counting Fos+ neurons with unbiased stereology

The optical fractionator probe in StereoInvestigator (MBF Biosciences, Williston VT) was used to estimate the overall number of Fos- and Fos+ neurons in each region of interest. This approach was necessary to calculate the proportion of neurons activated by the stimulus as opposed to the areal density of Fos+ cells. Areal density measurements are potentially confounded by sex differences in neuron number and/or cell density.

To begin the analysis, an investigator traced the outlines of the region of interest under 2.5x brightfield illumination using the Swanson (1992) and Paxinos and Watson (1998) atlases as guides. Regions of interest in each hemisphere were counted separately. Neurons that fell within the inclusion lines of the counting frame were identified as either Fos+ or Fos-. A Fos+ neuron was identified by a black DAB precipitate in its nucleus; Fos- neurons had a clear nucleus, and a lightly stained cytoplasm (Fig. 1A, inset). Neurons were distinguished from glia by their greater size, lighter staining, and smooth appearance. Fos labeling was not observed in glial cells. The counting frame size was 25 μm2 for all regions of interest whereas the x- and y-dimensions of the sampling grid varied for each region. Variable grid size was necessary to ensure that ~150 neurons were counted for every brain region, which usually resulted in a coefficient of error of ≤ 0.1. Counting frame height was 6 μm with 2 μm guard zones. The mean section thickness was 12 μm.

The optical fractionator estimates the number of neurons *N* as follows:

$$N=(\sum N)(1/ssf)(1/asf)(1/tsf)$$

$\sum N$ is the sum of neurons counted, ssf is the number of sampled sections divided by the total number of sections through the brain area, asf is the total area sampled by the counting frames divided by the total area of all sampled sections, and tsf is the thickness sampling fraction height of the disector divided by the average section thickness. The proportion of Fos+ neurons

was calculated by dividing the estimated total number of Fos+ neurons for each region by the estimated total number of neurons. The regional volume of each brain area was calculated by multiplying the total area of all sampled sections by the distance between each section (160 μm) and the thickness of each section (40 μm).

4.3.5 Statistics

Data from each hemisphere were first compared with a paired t-test or a repeated measures analysis of variance (ANOVA) to determine whether Fos+ neurons, regional volume, or the overall number of neurons was lateralized in a sex-specific and/or condition-specific manner. If laterality was not observed, data were collapsed across hemisphere and the mean value of each parameter was analyzed using a two-way ANOVA with experience and sex as the independent variables. ANOVAs within each sex followed by and comparisons with HC were used for additional analyses of the data. Results were considered statistically significant if $p < 0.05$.

4.4 Results

4.4.1 Behaviour

Juvenile rats were between the ages of P28 – P33 in this study, 10 days prior to the typical onset of adult-like levels of gonadotropin releasing hormone pulsatility in rats (Harris and Levine, 2003; Sisk et al., 2001). Thus, androgen and estrogen levels are undetectable, or very low and not sexually dimorphic (Cooke and Woolley, 2005a). The standard of exactly 10 acts of aggression (kicks, pins, and dominance postures) in ≤ 10 minutes ensured that males and females had virtually identical experiences both in terms of the overall number of aggressive acts received (t-test, P = 1.0) as well as the number of each type of aggressive act (t-tests, Ps range from 0.5 – 0.9).

Note that BC rats encountered the non-aggressive male in the soiled cage formerly occupied by an RA. Thus, the BC rats encountered similar olfactory cues to the JSS animals, as well as the unique odor of the non-aggressive adult.

4.5 Functional Neuroanatomy

4.5.1 Basolateral amygdala

The anterior subdivision of the basolateral amygdala (the BLAa; Swanson (1992)) was traced bilaterally in its entirety (Fig. 1A). The experience elicited sex-specific Fos responses in the juvenile rats (Fig. 1B). Males showed strong responses for the BC, whereas females were less selective in their responses. This sex-specific degree of selectivity led to a significant sex-by-experience interaction ($F_{2,29} = 4.0$, $p = 0.02$). One-way ANOVAs within each sex confirmed that the BLA of male, not female, rats responded selectively to the experiences; the effect of experience in males was statistically significant ($F_{2,16} = 5.29$, $p = 0.01$), whereas it was not among females. The effect of experience was caused by the significant reduction in Fos labeling by the BC experience ($t(9) = 4.0$, $p = 0.003$). In females, although HC the responses to BC and JSS were slightly elevated relative to HC, none were of sufficient magnitude or uniformity to show a distinct, statistically significant response.

Consideration of the absolute number of Fos+ neurons also indicated a sex-specific response to the experiences, although there was only a strong trend toward an interaction between sex and experience ($p = 0.050$; Fig. 1C). This trend prompted us to examine Fos responses within each sex using one-way ANOVA, which confirmed that males but not females were selective (Males: $F_{2,16} = 4.7$, $p = 0.02$; Females, $p > 0.05$). As with the proportional data, the BC experience caused a significant reduction in Fos expression relative to HC among males ($t(9) = 2.76$, $p = 0.02$), whereas BC and JSS tended to elevate Fos in females.

The regional volume of the BLA was similar in males and females (Fig. 1D), although males had a slightly larger left BLA than right and females did not show this weak lateralization. The number of neurons was significantly greater in the left than in the right BLA ($F_{1,33} = 6.97$, $p = 0.01$; Fig. 1E), and males clearly drove this effect, as there was a significant effect of laterality in males ($t(29) = 2.41$, $p = 0.02$), but not in females.

4.5.2 Bed nucleus of the stria terminalis, anterior subdivisions

The BSTad and BSTal, as defined in the Swanson atlas, were traced bilaterally in their entirety. The Fos response within these subregions was quite sparse, with no more than 0.01 – 0.02 of the neurons being Fos+. Moreover, none of the experiential conditions, the sex of the juvenile, or laterality influenced Fos expression (F values for the factors of sex, experience, and interaction, respectively: BSTad, $F_{2,25} = 0.1, 1.0, 0.3$, all $ps > 0.3$; BSTal, $F_{2,25} = 0.25, 0.02, 2.5$, all $ps > 0.10$). Morphometrically, the regional volume and cell numbers of the anterior subdivisions were similar across hemispheres. Thus, each parameter was averaged across the hemispheres for analysis. Regional volume and cell numbers were no different among males and females in the BSTal. In the BSTad, however, males had a slightly but non-significantly greater regional volume ($P = 0.06$) and a weak trend toward more neurons ($P = 0.12$).

Bed nucleus of the stria terminalis, principal subdivision

The BSTpr was traced bilaterally in its entirety (Fig. 2A). The proportion of neurons activated by the three experiences was comparable to the anterior BST, with values ranging from 0.014 to 0.028. JSS elicited the same proportion (0.028) of Fos+ neurons in males and females. In contrast to the anterior BST, however, JSS elevated Fos expression in both sexes, resulting in a significant main effect of experience ($F_{2,30} = 3.37$, $p = 0.04$; Fig. 2B). The response to JSS in

females, but not males, was of sufficient uniformity and magnitude as to be significantly different from HC ($t(10) = 2.78$, $p = 0.01$; Fig. 2B).

While BC elicited a near-identical Fos response (0.027) as JSS in females, the response to BC in males was identical in magnitude to that of HC. Thus, the pattern of responses, as reflected by the mean proportion of Fos+ neurons, was sex-specific: Males had the greatest Fos response to JSS, whereas females had the greatest Fos response to both JSS and BC.

When the overall number of Fos+ neurons was examined, the trends apparent in the proportion data became highly significant. The response in the BSTpr to the three conditions resulted in a significant main effect of experience ($F_{2,28} = 11.06$, $p = 0.0003$; Fig. 2C), and a significant interaction of sex with experience ($F_{2,28} = 5.98$, $p = 0.006$). While males showed a strong and selective response to JSS ($t(9) = 2.57$, $p = 0.03$), females responded equally to both BC and JSS experiences, relative to the handled condition (HC vs. BC, $t(9) = 3.2$, $p = 0.01$; HC vs. JSS, $t(10) = 4.87$, $p = 0.0006$).

Because cell counts and regional volumes were not lateralized, the average unilateral number of neurons and average unilateral regional volume in the BSTpr was compared. These analyses indicated that juvenile females have a significantly smaller regional volume than males ($t(29) = 2.14$, $p = 0.03$) as well as fewer neurons ($t(29) = 2.10$, $p = 0.04$).

4.5.3 Paraventricular nucleus of the hypothalamus

The PVN was bilaterally traced in its entirety (Fig. 3A). Counts of Fos+ neurons revealed that the PVN was responsive to the experiences, both in terms of the proportion of Fos+ neurons (Effect of experience: $F_{2,31} = 7.4$, $p = 0.002$; Fig. 3B) and in their overall number (Effect of experience: $F_{2,31} = 6.1$, $p = 0.005$; Fig. 3C).

A weak trend toward an interaction between sex and experience ($p = 0.1$) in the proportion of Fos+ cells prompted us to examine the data within each sex with ANOVA. This revealed that males largely accounted for the effect of experience (Effect of experience: Males, $p = 0.002$; Females, $p = 0.3$). The benign control suppressed Fos in the PVN of males. This impression was confirmed by a contrast within the males, which showed that the difference between HC and BC was statistically significant ($t(10) = 2.35$, $p = 0.04$). In contrast, males' Fos response to JSS, while greater than HC, was nonetheless statistically indistinguishable the control.

A similar effect was seen in the absolute number of Fos+ neurons: There, a one-way ANOVA in males showed a main effect of experience ($F_{2,17} = 7.35$, $p = 0.005$), while an ANOVA in females revealed no such effect. Contrasts within males confirmed that BC suppressed Fos relative to HC ($t(10) = 2.3$, $p = 0.03$), and that while JSS elevated Fos, it was not significantly greater than HC. Morphometrically, the PVN was neither lateralized nor sexually dimorphic in terms of neuron number (Fig. 3C) or regional volume (Fig. 3D) (all p's > 0.15).

4.6 Discussion

It is well established that the amygdala, BST, and PVN participate in the response to chronic stress in adult animals (Herman et al., 2003; Sawchenko et al., 2000). Moreover, there are reports that the PVN in particular responds to chronic stress in a sexually dimorphic manner (Dalla et al., 2008; Figueiredo et al., 2002; Goel and Bale, 2010; Iwasaki-Sekino et al., 2009; Sterrenburg et al., 2012). However, few studies have addressed sex differences in the prepubertal animal or how males and females respond to a single acute stressor. The present study supports the hypothesis that JSS elicits sex-specific neural activity. However, rather than females

encoding JSS with a greater proportion of neurons in each brain region as we had anticipated, females showed less stimulus selectivity than males.

Below, we discuss the significance of the present results at a neural circuit level in an effort to understand the events that underlie the encoding of JSS and their potential relevance to outcomes later in life. Although Fos is widely accepted as a marker of transcriptional activity at the cellular level, interpretation is not straightforward because its mRNA is induced by many factors including growth factors, neurotransmitters, as well as action potential discharges (Sagar et al., 1988). Thus, elevated activity may represent the transduction of a neurotrophin, the stimulation of the neuron by transmitter, and/or the activity of excitatory and inhibitory neurons.

4.6.1 The PVN

The PVN is the final common pathway through which stress-related signals are translated into the activation of sympathetic premotor neurons and the release of CRF and vasopressin into the portal vasculature. Not surprisingly, the PVN showed the strongest response to the three experiences among the examined regions, with approximately 12% of neurons in males activated by JSS and 9% in females. *c-fos* mRNA is strongly correlated with CRF expression in the PVN (Loughlin et al., 2006), making the presence of Fos+ neurons there an indicator of JSS-induced CRF release and elevated CORT.

Although JSS activated the PVN in males and females, the responses to the other conditions were sexually dimorphic. In females, JSS and BC increased Fos expression to a similar extent, resulting in a response profile that was less selective than in males. In contrast to females, the PVN of males responded selectively to the BC condition, showing a significant reduction in Fos expression relative to the handled control.

The selective response to the BC condition in males suggests that the friendly adult was anxiolytic relative to their encounters with a clean empty cage, and the aggressive adult. All juveniles were socially isolated during the week preceding this experiment, which is known to affect the development of social behaviour and the HPA axis in juvenile male rats (van den Berg et al., 1999). Perhaps the encounter with the benign control relieved what were otherwise stressful experiences: in one case, an empty cage completely devoid of conspecific odors, and the other, a cage occupied by an aggressive adult, plus his and his cagemates' odors.

One report suggests that several weeks of prepubertal social isolation impairs the immune response of males to a greater extent than in females (Hermes et al., 2006). If prepubertal social isolation is more stressful for males, and if an encounter with a friendly adult is anxiolytic, then this could potentially explain why the response to BC was more selective in males than in females.

4.6.2 The BLA

There is a striking similarity between the Fos response of the BLA and that of the PVN because BC elicited a strong reduction in Fos in males, whereas females' responses were non-selective. Although this might imply that the BLA and PVN are functionally connected, there is no evidence in the literature that these nuclei exchange direct afferents. A potential indirect route could be through the locus coereleus (Condes-Lara, 1998; Reyes et al., 2005) or medial prefrontal cortex, which influences the PVN via the ventrolateral BST and the BLA through direct excitatory projections (Figueiredo et al., 2003; McDonald, 1991).

The BLA - CeA pathway is considered the core system that mediates appraisal of appetitive and aversive cues and coordinating the appropriate motivational, affective, and neuroendocrine responses. As reported earlier, none of the experiential conditions elicited

appreciable levels of CeA Fos expression (Weathington et al., 2012b). The lack of correspondence between our Fos measurements in the BLA and CeA suggests that the canonical BLA-CeA pathway is not involved in encoding the BC and JSS experiences. Instead, the BLA may have encoded the state(s) elicited by JSS via its connections with the ventral striatum, anterolateral BST, or locus coereleus (Davis and Whalen, 2001).

Confirming previous reports, we did not observe a sex difference in neuron number or regional volume of the prepubertal BLA (Rubinow and Juraska, 2009). However, the number of neurons was greater in the left hemisphere than the right of males, but not in females. This, to our knowledge, is the first report of sex-dependent lateralization of cell number in the BLA. The significance of this finding is unclear, especially because lateralized Fos expression was not observed. Nonetheless, sex-linked lateralization is reminiscent of neuroimaging work by Giedd et al. (1996) who reported that the left amygdala was greater than the right in boys, and that of Cahill et al. (2001) who reported sex-linked lateralization of amygdala function in adults.

While sex-linked lateralization is a feature of the human central nervous system (Darlington, 2002; Geschwind and Galaburda, 1985), it is not commonly studied in rodents. Nonetheless, scattered reports indicate that the brain of rodents is lateralized in the hippocampus (Roof, 1993; Tabibnia et al., 1999), medial preoptic area (Holman and Collado, 2001), hypothalamus (Gerendai et al., 1995), and medial amygdala (Johnson et al., 2008) in a sex-dependent manner. Additionally, there is evidence that the function of the rodent amygdala is lateralized. For example, metabotropic glutamate receptor 5 is expressed in a lateralized fashion in the mouse amygdala (Kolber et al., 2010). Adamec et al. (2005) reported that predator stress potentiated afferents to the right but not the left amygdala. Baker and Kim (2004) report that right amygdala lesions impair the post-training retention of fear conditioning than lesions to the

left. It is important to note that these animal studies used males exclusively, precluding the possibility of observing a sex-dependent lateralization of amygdala function.

4.6.3 The BST

The BSTpr demonstrated a pattern of activity distinct from the BLA and PVN. While males responded selectively to JSS, females' BSTpr responded to both the benign male and the aggressive resident. Without a female stimulus animal in this study, we cannot determine whether the female responded to the sex of the stimulus animals and/or to social experience *per se*. Lesion studies have shown that the posterior BST mediates opposite sex odor preference in male hamsters and rats (Been and Petrulis, 2012; Liu et al., 1997) and sexual solicitation in female hamsters (Martinez and Petrulis, 2011). This suggests that the posterior BST participates in the appraisal of sexually relevant social cues. It may be that the stimulus males elicited both aversive and appetitive states that were mediated by separate microcircuits within the BSTpr.

The heterogeneity of the BST poses a challenge for integrative accounts of its role in behaviour. For example, lesions to its anterior subnuclei reduce HPA output, whereas lesions to the BSTpr increase it (Choi et al., 2007). While the BSTpr is sexually dimorphic and intensely expresses gonadal steroid receptors (Forger et al., 2004; Hines et al., 1992; Simerly et al., 1990), the anterior BST does not. Most importantly, the anterior BST is strongly interconnected with the central amygdala (Dong and Swanson, 2006a, b), while the BSTpr is strongly interconnected with the medial amygdala (Alheid and Heimer, 1996; Canteras et al., 1995; Dong and Swanson, 2004; Heimer et al., 1991). This connectivity may explain the functional heterogeneity observed here: Because the central amygdala was unresponsive to these conditions while the medial amygdala was selective for JSS in a sex-dependent manner (Weathington et al., 2012b), it is not surprising that the main targets of these areas would reflect a similar pattern of activity.

4.6.4 Relevance to sex-specific effects in adulthood

Our long-term goal is to understand why adult females seem to be more susceptible than males to the deleterious effects of JSS on mood disorder-like behaviour, which were manifest after 10 episodes (Weathington et al., 2012a). We cannot say with certainty whether the present findings have any bearing on the sex-specific effects described previously because we do not know whether a single JSS episode would induce a similar pattern of mood disorder-like states, or whether nine more episodes would result in similar Fos expression patterns as after acute JSS. However, it is clear that the female MePD, BLA, BSTpr, and PVN are less selective or discriminating of the three stimuli than males. What could be the behavioural significance of this? One possibility is that greater selectivity is identical to improved attention. There are reports of sex differences in selective attention in rats, including the work of Bayless et al. (2012) who reported that adult males had better performance in the 5-choice serial reaction time task under noisy conditions than females. A study by Risbrough et al. (2004) provides circumstantial support for sex differences in attention. They used psychopharmacology to show that stimulation of the type-2 corticotrophin releasing factor receptor (CRF_2) enhanced the impact of sensory information on defensive behaviour, specifically by increasing pre-pulse inhibition of fear-potentiated startle. And we have found that juvenile males have greater CRF_2 binding in the BSTpr than females (Weathington, Hamki, & Cooke, *under revision*). If the sex difference in CRF_2 binding influences sensory processing in juvenile rats, then this could be a mechanism by which males have greater stimulus selectivity than females.

In conclusion, our investigation of Fos expression revealed differential processing between males and females. Across examined brain regions, females were less able to discriminate between stimuli, contrary to the prediction that they would simply display a greater proportion of Fos positive nuclei than males. This finding may help to pinpoint the neural

mechanisms that lead to the disproportionate number of females affected by mood disorders despite comparable exposure to stressful experiences. Future studies could employ the use of female stimulus animals to confirm that observed Fos activation was a result of the social experience and not the sex of the aggressor.

4.7 Acknowledgments

The authors acknowledge Carley Shulman for their invaluable contributions to this project.

4.8 Chapter 4 Figures

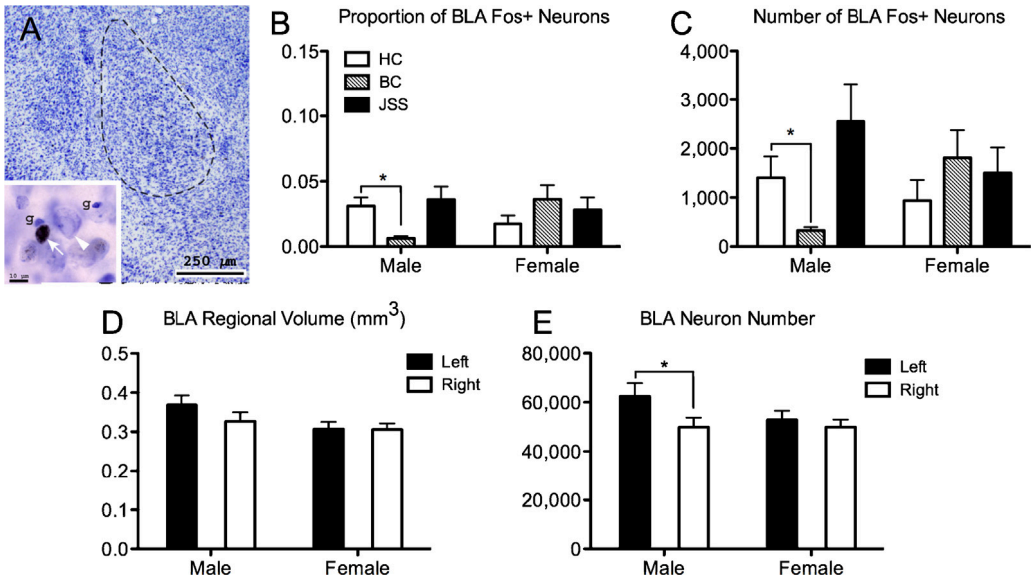

Figure 10 Fos expression in the BLA following juvenile social subjugation, benign control, or handling experience

A. Photomicrograph of the anterior basolateral amygdala (BLA). Inset: High magnification photomicrograph showing Fos+ neuron (arrow), Fos- neuron (arrowhead), and two glial cells ('g'). *Note:* The scale of the Y-axis in Figs. 1 and 2 is matched to Fig. 3. **B.** Sex-specific response in juvenile males and females. The Fos response in the BLA of males was selective to

the benign control (BC) (* t(9) = 4.0, p = 0.003). No such selectivity was apparent in females. **C.** The overall number of Fos+ neurons showed a greater degree of selectivity among males, with BC once again suppressing activity relative to HC (* t (9) = 2.76, p = 0.02). **D.** No sex difference in BLA regional volume. **E.** Sex-specific laterality in neuron number. Males have more BLA neurons in the left hemisphere than in the right (* t(29) = 2.41, p = 0.02); females lack this lateralization.

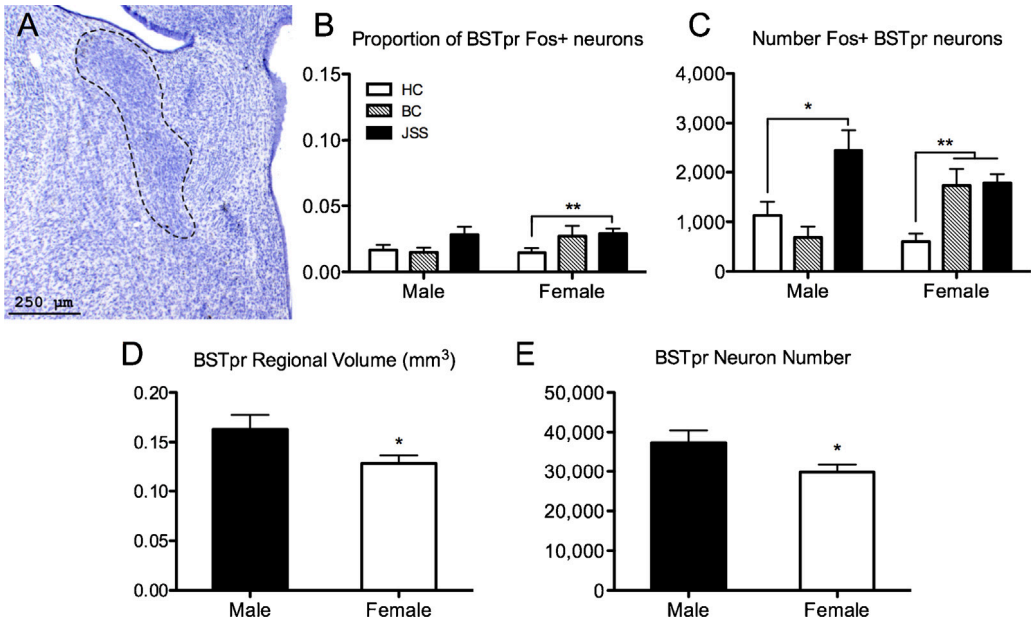

Figure 11 Fos expression in the BSTpr following juvenile social subjugation, benign control, or handling experience

A. Photomicrograph of the columnar portion of the principal subdivision of the bed nucleus of the stria terminalis (BSTpr). **B.** The proportion of Fos+ neurons in the male and female BSTpr in response to JSS was similar in magnitude between the sexes, and statistically significant in females (** t(10) = 2.78, p = 0.01; Fig. 2B). **C.** The overall number of Fos+ neurons was clearly sex-specific: Males' response was selective for JSS (* t(9) = 2.57, p = 0.03), whereas females' response was selective for the social experience *per se* and/or the sex of the stimulus animals (**

both ps < 0.01). **D.** BSTpr regional volume was significantly greater in prepubertal males than females (* t(29) = 2.14, p = 0.03), as was the overall number of neurons (* t(29) = 2.10, p = 0.04).

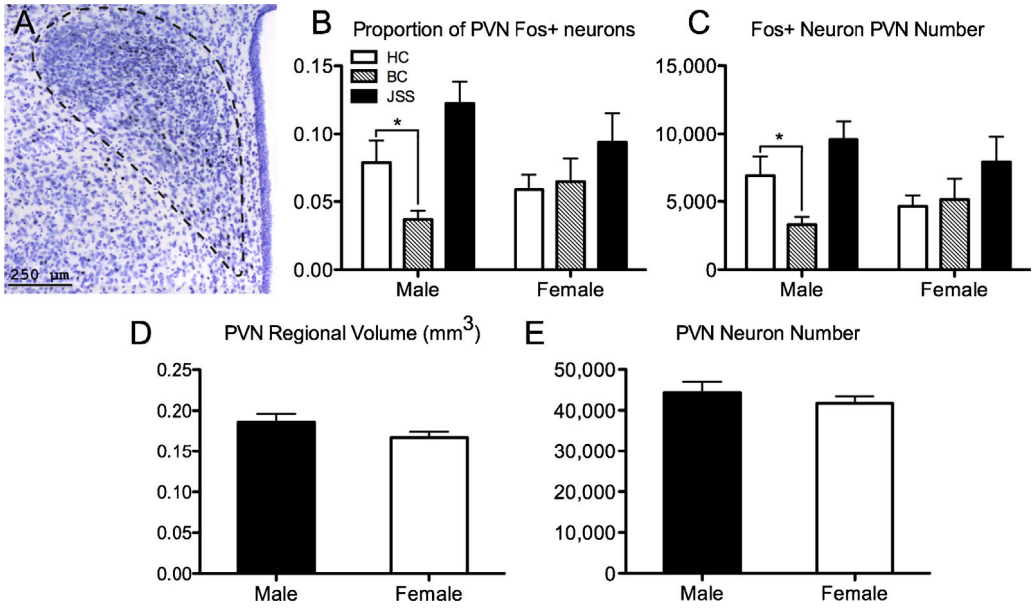

Figure 12 Fos expression in the PVN following juvenile social subjugation, benign control, or handling experience

A. Photomicrograph of the paraventricular nucleus (PVN) showing numerous Fos+ neurons. **B - C.** In both the proportion and the overall number of Fos+ neurons, males' response was selective for BC (* both ps < 0.05), whereas females' responses to BC and JSS were of similar magnitude and thus non-selective. **D – E.** Regional volume and neuron number were both sexually monomorphic in the juvenile rats.

5 CHAPTER FIVE: CORTICOTROPIN-RELEASING FACTOR RECEPTOR BINDING IN THE AMYGDALA CHANGES ACROSS PUBERTY IN A SEX-SPECIFIC MANNER

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5.1 Abstract

Corticotropin-releasing factor receptor types 1 (CRF_1) and 2 (CRF_2) have complementary roles controlling the hypothalamic-pituitary-adrenal (HPA) axis. Because corticotropin-releasing factor receptors are expressed in sex steroid-sensitive areas of the forebrain, they may contribute to sex-specific patterns of stress sensitivity and susceptibility to stress-related mood disorders, which are more frequent in women. To determine whether corticotropin-releasing factor receptors vary as a function of age and/or sex, we measured receptor binding in the amygdala of prepubertal and adult rats. Both receptor subtypes demonstrated age- and sex-specific binding patterns. In the basolateral amygdala and the posteroventral medial amygdala, CRF_1 binding decreased in males and increased in females after puberty, while CRF_2 binding increased in males and was unchanged in females. In the posterodorsal medial amygdala, CRF_1 binding was unchanged across puberty, whereas CRF_2 binding increased across puberty far more in males than in females. Overall binding was lowest in the central amygdala where CRF_2 binding increased across puberty only in males. Thus, in all four examined areas across prepuberty to adulthood, CRF_2 binding increased far more in males than in females and resulted in

significantly more binding in adult males than in adult females. These sex-specific developmental patterns are consistent with sex differences in HPA responsiveness and may thus contribute to sex differences in mood disorder susceptibility.

5.2 Introduction

Rising levels of sex steroid hormones during puberty coincide with two major divergences between the sexes; cortisol levels and the prevalence of stress-related mood disorders both increase in women relative to men (Altemus, 2006). These clinical findings have led to the suggestion that circulating sex steroid hormones contribute to sex differences in mood disorders by inducing plasticity in areas that regulate mood and the HPA axis (Becker et al., 2007). Moreover, because the brain is particularly plastic during childhood and puberty, adverse experiences around this time have a disproportionate effect on mood disorder risk. Indeed, being the victim of child abuse is the most significant environmental risk factor for the development of mood disorders (Heim and Nemeroff, 2001; Neigh et al., 2009)

Using an animal model of child abuse, we found that adult female rats that had been subjected to repeated juvenile social subjugation (JSS) have significantly higher stress-evoked corticosterone (CORT) levels and more severe mood disorder-like behaviors than controls or JSS males that received the same treatment (Weathington et al., 2012a). We then identified JSS-selective neural activity in the posteroventral medial amygdala (MePV) and in the posterodorsal medial amygdala (MePD) of both sexes (Jill et al., 2012). The central nucleus of the amygdala (CeA) showed virtually no response to JSS or any other condition, possibly because it is less responsive than the medial amygdala (MeA) to psychogenic stressors (Dayas et al., 1999).

CRF receptors are concentrated in regions such as the amygdala that regulate arousal, mood, and the HPA axis, and both subtypes are expressed in discrete amygdaloid nuclei

(Chalmers et al., 1995; De Souza et al., 1984). Numerous studies show that CRF_1 activation initiates the neuroendocrine and behavioral responses to a stressor whereas CRF_2 activation decreases stress responses (Bale et al., 2002). For example, CRF_1 agonists increase anxiety-like behavior in rats (Takahashi, 2001) and CRF_1 knock-out mice are less anxious and have blunted stress-evoked CORT responses (Bale et al., 2002; Timpl et al., 1998). Conversely, the CRF_2 -specific agonist urocortin 3 decreases anxiety-like behavior (Valdez et al., 2003) and CRF_2 knock-out mice display more anxiety-like behavior and a delayed recovery of CORT levels to the pre-stress baseline (Bale et al., 2000; Preil et al., 2001). The importance of CRF receptors in regulating anxiety and the HPA axis as well as interrelationships between gender, sex steroid hormones, and mood disorder susceptibility led us to hypothesize that CRF receptor expression is sexually dimorphic in the amygdala.

Thus, with *in situ* receptor-binding autoradiography followed by measurements of optical density, we compared CRF_1 and CRF_2 binding in the MeA, CeA, and basolateral amygdala (BLA) in prepubertal and adult, male and female rats. We report here that CRF receptor binding changes across puberty in a subtype-, region-, and sex-specific manner.

5.3 Materials and Methods

5.3.1 Autoradiography

Twenty-four prepubertal (P30) and adult (P98) male and female Long-Evans rats (Charles River) were group housed in standard laboratory cages on a reverse light cycle (lights on at 0700 am) and were provided food and water ad libitum. Animal procedures used in this study met the standards outlined in the *N.I.H. Guide for the Use of Laboratory Animals* and were in accord with the Georgia State University Institutional Animal Care and Use Committee. All rats were sacrificed by rapid decapitation between 1000 and 1300 and their brains rapidly frozen

in isopentane and cryosectioned at 20 μm onto slides. Slides were stored at -80º C until processing with competitive receptor-binding autoradiography to visualize CRF_1 and CRF_2 binding (Lim et al., 2005). Slides were brought to room temperature, fixed in 0.1 M paraformaldehyde (pH 7.4), and washed in 50 mM Tris buffer before a 2 hr incubation in 4 mL custom-built binding chambers containing 0.2 nM ^{125}I sauvagine (Perkin Elmer), which binds with equal affinity to CRF_1 s and CRF_2 s. To visualize CRF_1 binding, 500 mM Astressin 2B (Sigma-Aldrich), a highly selective CRF_2 antagonist, was added to the binding chambers. To visualize CRF_2 binding, 500 mM CP-154,526 (Tocris), a highly selective CRF_1 antagonist, was added to the binding chambers. After a 2 hr incubation, slides were washed in 50 mM Tris buffer + $MgCl_2$, rinsed in deionized H_2O, and air-dried. Slides were then affixed to a backboard and placed in a light-tight box where Kodak Biomax MR film was exposed to the slides for 90 hr after which films were developed and fixed.

5.3.2 Densitometry

Standard curves for each film were created with densitometry readings of ^{125}I microscale standards (ARC) acquired with Scion Image (NIH and Scion Corp). Receptor binding density was calculated by subtracting the background signal in each animal from the reading from the region of interest (Fig. 1A), both of which were measured in a 0.35 mm^2 square. Background measurements were taken from the lateral dorsal thalamus for CRF_1 and from the dorsal striatum for CRF_2, areas that showed no CRF receptor binding. A third series of slides was stained with Neutral red and used for anatomical reference. Positive controls were incubated in 0.2 nM ^{125}I sauvagine without antagonists and, as expected, ligand binding was observed in regions known to express mRNA for both receptors. Negative control slides were incubated in 0.2 nM ^{125}I

sauvagine, 500 mM CP-154,526, and 500 mM astressin 2B and the addition of both antagonists to the binding solution blocked all binding.

Heat maps (Fig. 1B-C) were created in Photoshop. Representative brains were selected as those whose densitometry measurements most closely matched the mean for each receptor type within each group.

5.3.3 Statistics

Data were analyzed with a two-way ANOVA where age and sex served as the between-subject variables. Post-hoc Student's t-tests were conducted to assess effects of age within each sex or of sex within each age.

5.4 Results

5.4.1 Patterns of amygdala CRF receptor binding

As shown in Fig. 1 B-C, CRF receptor binding was distributed in discrete subnuclei throughout the amygdala. CRF_1 binding was most intense in the BLA whereas CRF_2 binding was comparatively weak there. On the other hand, both receptor ligands bound quite avidly in the MeA: The intensity of CRF_1 binding in the MePV was second only to that in the BLA. CRF_2 binding in the MePV was half as intense but nonetheless still apparent in the autoradiogram. Binding of both receptor subtypes became progressively weaker in the MePD along a ventro-dorsal gradient. Neither receptor was expressed to any remarkable extent in the CeA. Incidental observations included intense CRF_1 binding in layer IV of the frontal cerebral cortex and very intense CRF_2 binding in the choroid plexus, cortical nucleus of the amygdala, lateral septum, bed nucleus of the stria terminalis, and ventromedial hypothalamus.

5.4.2 CRF receptor binding in juvenile and adult, male and female rats

There was no hemispheric laterality in binding of either subtype and binding did not vary across hemisphere as a function of sex, age, or as any interaction of hemisphere with sex or age, or sex and age (all p's ≥ 0.06). Therefore, the data were pooled across hemispheres and analyzed.

5.4.3 Basolateral amygdala

CRF_1 binding in the BLA (Fig. 2A) was indistinguishable between the sexes before puberty. By P98, however, CRF_1 binding had decreased in males and increased in females (sex-by-age interaction: $F_{1,17} = 4.88$, $p = 0.04$). This finding was supported by a sex difference in adults that favored females ($p = 0.0009$). Although BLA CRF_2 binding was much lower than CRF_1, its maturation pattern was nonetheless clearly sex-specific (Fig. 2B): CRF_2 binding increased in males and decreased slightly in females. This age-by-sex interaction was statistically significant ($F_{1,17} = 7.23$, $p = 0.01$) and was reinforced by the presence of a sex difference that favored adult males ($p = 0.04$) and by an increase in CRF_2 binding in males with age ($p = 0.002$).

5.4.4 Posteroventral medial amygdala

As in the BLA, CRF_1 binding in the MePV (Fig. 2C) changed dramatically with age and the rat's sex determined the direction of that change. Although CRF_1 binding was indistinguishable between the sexes before puberty at P30, it had become sexually dimorphic by P98: Binding increased in females and decreased in males (effect of sex: $F_{1,17} = 6.5$, $p = 0.02$; sex-by-age interaction: $F_{1,17} = 6.3$, $p = 0.02$). CRF_1 binding was significantly greater in adult females than in adult males ($p = 0.006$), and was considerably greater overall relative to CRF_2 binding (Fig. 2D). CRF_2 binding showed a strong trend toward an age-dependent increase ($p = 0.057$) as seen in the BLA. This developmental trend was clearly driven by males, whose CRF_2 binding increased with age ($p = 0.02$), whereas it was unchanged in females ($p = 0.7$).

5.4.5 Posterodorsal medial amygdala

CRF_1 binding in the MePD was indistinguishable between the sexes both before and after puberty (Fig. 2E). In contrast, CRF_2 binding increased with age (Fig. 2F; $F_{1,17} = 14.98$, $p = 0.001$) and did so to a far greater extent in males. This impression was confirmed by a significant increase in binding across age in males only (males: $p = 0.0002$; females: $p = 0.30$).

5.4.6 Central amygdala

CRF_1 ligand binding (Fig. 2G) was lower in the CeA as compared to the other areas and did not vary as a function of age or sex or as an interaction between those factors. CRF_2 binding (Fig. 2H) was virtually undetectable in prepubertal rats, yet it increased significantly between P30 and P98 (effect of age: $F_{1,17} = 18.89$, $p = 0.0004$) to a far greater extent in males than in females (sex-by-age interaction: $F_{1,17} = 6.52$, $p = 0.02$; males' effect of age, $p = 0.0008$; females, $p = 0.24$).

5.5 Discussion

Our observations of CRF receptor binding in the amygdala closely parallel the reports of mRNA expression by Chalmers e*t al.* (1995) and VanPett *et al.* (2000) (Chalmers et al., 1995; Van Pett et al., 2000). Both subtypes were expressed to some extent in every region examined and each had distinct binding levels and developmental trajectories within each area. Overall, these data indicate that with the achievement of puberty and/or physical maturation, males and females diverge significantly in CRF receptor binding.

Before puberty, binding densities were indistinguishable between the sexes. Afterwards, one or both subtypes demonstrated a sex-specific alteration in binding such that a sex difference emerged in every subregion we examined. In the BLA and MePV, CRF_1 became sexually dimorphic after small changes in each sex additively produced a sex difference. Although there

is no evidence that estrogen regulates CRF_1 expression directly, it does upregulate CRF mRNA transcription (Lunga and Herbert, 2004), thus increasing HPA sensitivity to stressors (Viau and Meaney, 1991; Young et al., 2001). The small increase in CRF_1 binding in females may thus have been due to increased endogenous ligand availability. Nonetheless, the greater abundance of CRF_1 in adult females is consistent with their greater HPA sensitivity and risk of mood disorder-like behaviors.

In contrast to CRF_1, CRF_2 in males increased with age in every region examined. This was to such an extent in the BLA that binding levels were sexually dimorphic at P98. Weiser et al. (Weiser et al., 2008) reported that the non-aromatizable androgen 5a-dihydrotestosterone increases CRF_2 mRNA in several forebrain areas, indicating that the androgen receptor probably mediates the post-pubertal increase CRF_2 binding in the amygdala. Given the role of CRF_2 in suppressing HPA activity, greater CRF_2 binding in adult males is consistent with their lower stress responsiveness. Furthermore, if greater CRF_2 binding also increases at puberty in boys, it could be an important factor that protects them from stress-related mood disorder development.

The MeA intensely expresses mRNAs for the androgen receptor and estrogen receptors, making it exquisitely sensitive to adult circulating androgens (Cooke, 2006). In contrast, sex steroid hormone receptors are only lightly expressed in the CeA and BLA (Shughrue et al., 1997; Simerly et al., 1990). Nonetheless, their presence confers them with the ability to directly respond to sex steroid hormones; indeed, the number of CeA neurons was recently found to be sexually dimorphic in juvenile rats (Jill et al., 2012). If sex steroid hormones regulate CRF_2 binding by acting on extant neurons, then the increased binding must be due to more receptors per cell and/or greater affinity of the receptor for ligand. Alternatively, the effect would also be explained if the number of CRF_2-expressing cells were to increase during puberty. More CRF_2-

expressing cells would dilute the population of CRF_1 -expressing cells, shifting the CRF_2 /CRF_1 ratio in the male amygdala in an HPA-suppressive and anxiolytic direction. In line with this idea, pubertal androgens reportedly foster neuronal and glial proliferation in the MeA and elsewhere in the amygdala (Ahmed et al., 2008).

The present study demonstrates that CRF receptors undergo maturational changes consistent with the pubertal differentiation of the stress response system in males and females and the hypothesized role of this sex difference in the predisposition of women to develop stress-related mood disorders. CRF_1 binding is greater in the amygdala of adult females than males, whereas CRF_2 binding is greater in adult males than females. If CRF_1 s are normally anxiogenic and activate the HPA axis and if CRF_2 s are normally anxiolytic and inhibit the HPA axis, this sex-specific pattern of changes in CRF receptor expression across puberty could contribute to sex differences in HPA axis activity and the propensity to develop mood disorder-like behaviors

5.6 Acknowledgments

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5.7 Chapter 5 Figures

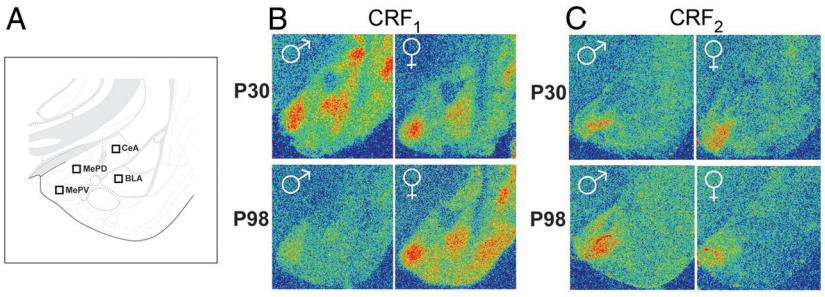

Figure 13 Location of sampling sites and representative heat maps of CRFR binding in the amygdala

A. Atlas plate indicating sampling size and locations. **B-C.** Heat maps of CRF receptor binding from representative animals of each group. **B.** CRF_1 binding. **C.** CRF_2 binding.

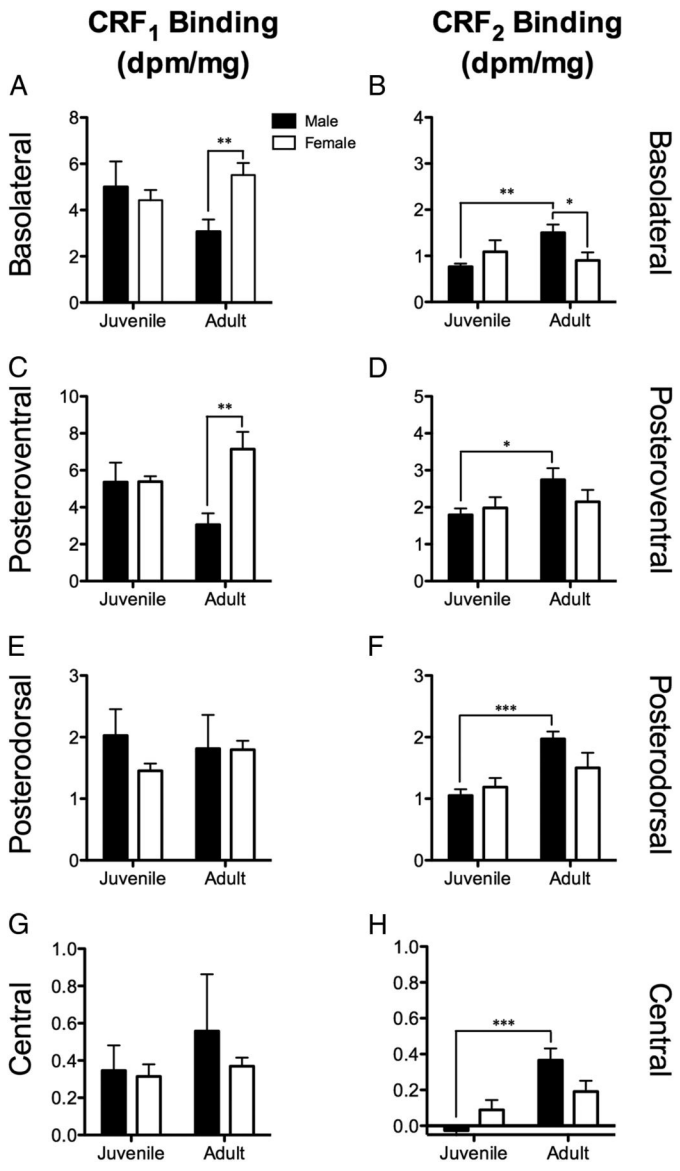

Figure 14 Mean (± SEM) binding (decays min^{-1} mg^{-1}) in four regions of the amygdala in young and old male and female rats

A. BLA CRF_1 binding was greater in adult females. **B.** BLA CRF_2 binding was greater in adult males than females. **C.** MePV CRF_1 binding was significantly greater in adult females. **D.** MePV

CRF_2 binding increased with age in males only. **E.** No sex difference or effects of age in MePD CRF_1 binding. **F.** MePD CRF_2 binding increased with age in males only. G. No sex differences or effets of age in CeA CRF_1 binding. **H.** CeA CRF_2 binding increased with age in males only (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

6 CHAPTER SIX: SEX- AND REGION-SPECIFIC PUBERTAL MATURATION OF THE FOREBRAIN CRF RECEPTOR SYSTEM IN THE RAT

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6.1 Abstract

One of the most reliable findings in psychiatry is in the incidence of anxiety and depression. Beginning at puberty, women develop mood disorders twice as often as men. Because corticotrophin releasing factor (CRF) receptors are implicated, we compared CRF receptor binding in pre- and postpubertal rats. In each brain area, CRF receptor binding was sexually dimorphic, but no two areas were alike in the way the sexes differed. In the nucleus accumbens and olfactory tubercle, CRF_1 binding was initially the same in juveniles, but became greater in adult females. In piriform cortex, CRF_1 binding increased in females and decreased in males, again becoming sexually dimorphic. CRF_1 binding in the anterior cingulate was greater in females than in males at both ages. In CA3, CRF_1 binding was greater in males before puberty but decreased during puberty, abolishing the sex difference. CRF_2 binding in the posterior bed nucleus of the stria terminalis was greater in males irrespective of age. In contrast, in each of three subdivisions of the lateral septum, females had greater CRF_2 binding than males as juveniles, or as juveniles and as adults. CRF_2 binding in the ventromedial hypothalamus was the

same in juveniles, but binding levels increased in males, leading to an adult sex difference. Thus, eight CRF receptor-expressing areas displayed eight distinct sex differences. These results show that sex differences pervade the CRF receptor system in juvenile and adult rats, and the mechanisms that control them are likely to be sex-, region-, and subtype-specific.

6.2 Introduction

Considerable evidence implicates corticotrophin releasing factor (CRF) receptors in the pathogenesis of stress-related mood disorders such as depression and anxiety. Mood disorders are diagnosed twice as often in women as in men (Altemus, 2006; Kessler, 2003), a sex difference that emerges during puberty and transcends culture, geography, and socio-economic status (Tolin and Foa, 2006). Given the associations between gender, CRF receptors, and the susceptibility to mood disorders, it is reasonable to ask whether CRF receptors mediate sex-specific functions and/or are expressed in a sexually dimorphic manner in stress-related brain regions. Both type 1 and 2 CRF receptors are expressed in forebrain areas that mediate anxiety and depression, such as the nucleus accumbens, anterior cingulate cortex, lateral septum, bed nucleus of the stria terminalis, the hippocampus, and amygdala. Each of these areas expresses estrogen receptors and/or the androgen receptor, or has afferents that express them (Kritzer, 2004; Kritzer, 2002; Shughrue et al., 1997; Simerly et al., 1990), making sex steroid hormones potentially capable of regulating CRF receptors. If progress is to be made in understanding sex-typical patterns of mood disorder prevalence, it is important to determine whether the functional expression of CRF receptors in these regions is influenced by gender and/or by age.

Mood disorders are often accompanied by dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis; elevated CRF in the cerebrospinal fluid of depressed patients was a seminal early finding (Nemeroff et al., 1984). Abnormally high levels of CRF act via type 1 CRF

receptors (CRF_1) to drive the pituitary to release more adrenocorticotropic releasing hormone, thereby chronically elevating cortisol and causing patients to fail the dexamethasone suppression test, indicating dysregulation of the HPA axis (Nemeroff, 1988; Nemeroff et al., 1992). In addition to its role as a releasing hormone, CRF also acts centrally via CRF_1s to coordinate the behavioral response to an acute stressor. CRF_1 agonists promote anxiety-like behavior such as fear-potentiated startle (Swerdlow et al., 1986), while CRF_1 antagonists reduce anxiety and depression-like symptoms (Takahashi, 2001). Overexpression of CRF produces an anxious phenotype in mice (Dedic et al., 2012), and conditional knock-out of *crfr1* from the forebrain of adult mice reduced anxiety and stress-induced cognitive deficits (Müller et al., 2003; Wang et al., 2011). The majority of studies indicate a primarily anxiogenic role for CRF_1s, although the concept of a unipolar effect by this receptor has been recently challenged by site-specific manipulations of CRF expression (Janssen and Kozicz, 2013). Whereas the role of CRF_1 s in mood and HPA output is fairly clear, the role of extra-hypothalamic CRF_2 in neuroendocrine control and behavior is less straightforward. Some studies indicate a role for CRF_2 s in anxiolysis and the adaptation of the HPA axis to a stressor, whereas others indicate the opposite (Bakshi et al., 2007; Bale et al., 2000; Jamieson et al., 2006; Sajdyk et al., 1999; Skórzewska et al., 2011; Valdez et al., 2003; Zhao et al., 2007).

This controversy suggests that type 1 and type 2 CRF receptors have overlapping or complementary functions in controlling behavioral and neuroendocrine stress responses and do not simply oppose one another. Adding further nuance to the respective roles of CRF receptors is the work of Risbrough et al. (2004), who showed that while CRF_1 and CRF_2 agonists both enhanced HPA output, they had opposing effects on pre-pulse inhibition of fear potentiated startle: CRF_1 stimulation reduced pre-pulse inhibition, whereas CRF_2 stimulation increased it,

making those treated with a CRF_2 agonist less likely to startle. These findings led them to suggest that CRF_1 plays a key role in mediating acute responses to threat, whereas CRF_2s facilitate survival of longer-term threats by promoting greater cognitive flexibility.

Considering the important role of the amygdala in many psychiatric conditions including mood disorders, we previously compared CRF_1 and CRF_2 binding in pre-pubertal (P30) and post-pubertal (P98) males and females (Weathington and Cooke, 2012). In juvenile rats, there were no sex differences in binding for either receptor subtype. However, by P98, CRF_1 binding in the medial (MeA) and basolateral (BLA) subdivisions of the amygdala had increased in females and decreased in males, leading to a sexual dimorphism in binding density, favoring females. In contrast, CRF_2 binding in the BLA, MeA, and central amygdala (CeA) increased across puberty selectively in males, leading to another sex difference, this time favoring males.

Notwithstanding the ambiguity about CRF receptors' respective roles in anxiety-like behaviors, the data suggested that these patterns of CRF receptor maturation could contribute to sex-specific patterns of mood disorder susceptibility, particularly because the changes coincide with puberty. However, the amygdala is but one node in a much larger network that contributes to mood and anxiety. If the maturational patterns observed in the amygdala were true of other brain regions, this would add support to the idea that greater CRF_1 binding in adult females and greater CRF_2 binding in males is related to the emergence of sex differences in mood disorder prevalence during puberty.

Thus, the purpose of the present study was to determine the maturational pattern of CRF receptor binding in several extra-amygdala areas of the forebrain. We examined CRF_1 binding in the shell of the nucleus accumbens, the olfactory tubercle, the anterior cingulate cortex, the dentate gyrus and CA3 layers of the dorsal hippocampus, and the paraventricular nucleus of the

hypothalamus. We measured CRF_2 binding in three regions within the lateral septum, in the posterior bed nucleus of the stria terminalis, and the ventromedial hypothalamus. All of these regions express CRF receptor mRNA (Chalmers et al., 1995; Van Pett et al., 2000) and contribute to anxiety-like states, and processing stressors. Unlike the pattern in the amygdala, however, the present results indicate that CRF receptor binding matures in a sex- and region-specific manner that defies classification as simply being greater in one sex or another.

6.3 Materials and Methods

The Institutional Care and Use Committee at Georgia State University approved all of the procedures involving animals. Long-Evans rats were obtained from Charles River Laboratories (Germantown, MD), group-housed in a reversed 12:12 light-dark cycle (lights off at 0700), and provided food and water *ad libitum*.

6.3.1 Autoradiography

Postnatal day (P) 30 and P98 male and female rats (n = 6 / group) were decapitated between 1000 and 1300 hr, their brains were rapidly removed, and frozen in isopentane. Brains were then stored at -80º C until sectioning. Brains were coronally sectioned at 20 µm on a cryostat and thaw-mounted onto slides in four series, resulting in a sampling interval of 80 µm. To visualize the binding of CRF_1 and CRF_2 receptors, we used competitive binding autoradiography, with empirically derived incubation times, as described earlier (Weathington and Cooke, 2012). Briefly, mounted sections were fixed, washed with Tris buffer, and incubated for 2 hrs in custom-made chambers containing 0.2 nM ^{125}I sauvagine (Perkin Elmer, Grayson, GA), which binds with equal affinity to CRF_1 and CRF_2. We visualized CRF_1 by adding 500 mM Astressin 2B, a highly selective CRF_2 antagonist (Sigma-Aldrich, St. Louis, MO), to the binding chambers. Astressin 2B binds with higher affinity to CRF_2s than ^{125}I sauvagine, thus leaving ^{125}I

sauvagine free to bind to and label CRF_1s. To visualize CRF_2 binding, we included 500 mM CP-154,526, a highly selective CRF_1 antagonist (Tocris, Bristol, UK), in the incubation buffer. CP-154,526 binds to CRF_1 with high affinity, thus outcompeting ^{125}I sauvagine for occupation of CRF_1 and leaving ^{125}I sauvagine free to bind to and label CRF_2 .

Additional sections were incubated in 0.2 nM ^{125}I sauvagine alone to verify its binding specificity (Fig. 1A). This resulted in binding signal within areas known to express CRF_1 mRNA and/or CRF_2 mRNA. To confirm the specificity of antagonist binding, slides were incubated in 500 mM CP-154,526 as well as 500 mM Astressin 2B, in addition to 0.2 nM ^{125}I sauvagine. As expected, this procedure blocked all binding and resulted in sections indistinguishable from those treated without any ligands (Fig. 1B).

After incubation, the slides were washed, rinsed with deionized water, and dried. They were then placed in a light-tight box and exposed to Kodak Biomax MR film for 90 hr.

6.3.2 Densitometry

We selected the nucleus accumbens shell (AcbS), olfactory tubercle (OT), anterior cingulate cortex (ACC), dorsal CA3 and dentate gyrus (DG), the dorsal, intermediate, and ventrolateral subregions of the lateral septum (LSd, LSi, and LSvl, respectively), the piriform cortex, posterior BST, VMH, and PVN for analysis and used Scion Image (NIH and Scion Corp) to measure the optical density of these regions. The optical density of each region was obtained by subtracting the background in each animal from the values obtained at the region of interest. Background regions were selected by their proximity to the region of interest and by the absence of any mRNA reported for that receptor. Each measurement was corrected by its own background reading. Thus, the corrected values are the relative optical density, not absolute density. For the dorsal CA3, background measurements were taken from the thalamus. For the

ACC, background signal measurements were taken from the corpus callosum. For the AcbS, LS, and posterior BST measurements, background signal measurements were obtained from the dorsal striatum. Background measurements were obtained from the lateral hypothalamus for the VMH and PVN.

For every region except the dorsal CA3, optical density measurements were obtained with a 0.25 mm^2 square centered over the region of interest. The dorsal CA3 was measured with a 0.20 mm^2 square. Neutral Red was used to stain an alternate series of sections for anatomical reference. The location of the measurement in each region is shown in the figures.

6.3.3 Data analysis

In each region of interest in each animal, we measured the optical density of the region of interest and background three times in each hemisphere across three serial sections. We then used the standard curve to calculate the receptor binding density, which is represented as disintegrations per minute per milligram of tissue (dpm/mg). This value is calculated with the standard curve, established from the ^{125}I microscale standards on each film. Thus, a particular decay rate corresponds to a particular optical density.

The data were normally distributed in each region except the VMH, so those data were log-transformed before further analysis. Because subcortical systems are sometimes asymmetrical across the cerebral hemispheres in terms of their structure and/or function, we initially examined the data for laterality effects within each group by comparing mean left – right values with paired t-tests. Laterality was not detected, so the data for each brain region in each group were averaged across hemispheres and analyzed with a two-way analysis of variance, with age and sex as the between-subjects variables and optical density as the dependent variable.

For data presentation, an animal with values closest to the mean for that group was selected. Images were captured and finalized with Adobe Photoshop software, although alterations were limited solely to applying a pseudocolored heat map to the image, cropping, and resizing. Otherwise no other modifications were performed on the images. Heat maps represent the range of pixel intensity within the brain section, and thus provide an accurate representation of autoradiogram signal. Bonferroni-corrected t-tests were used to assess effects of age within each sex, and sex differences within an age group. $P < 0.05$ was considered statistically significant.

6.4 Results

A graphical summary of the data and those from Weathington and Cooke (2012) is presented in the Table 1.

6.4.1 CRF_1 binding

6.4.1.1 Nucleus accumbens shell

CRF_1 binding clearly delineated the shell of the accumbens, forming a crescent shaped band reaching from the ventral tip of the lateral ventricle to the ventral margin of the caudate putamen (Fig. 2A). Binding decreased across puberty in both sexes, although the greatest decrease across age was in the males: They showed a significant reduction in binding ($t = 5.12$, $p = 0.00008$), whereas females did not. This male-specific reduction in binding density resulted in a statistically significant effect of age ($F_{1,15} = 19.54$, $p = 0.0005$) and a sex-by-age interaction ($F_{1,15} = 6.84$, $p = 0.01$).

6.4.1.2 Olfactory tubercle

The olfactory tubercle (Fig. 2A) showed a very similar pattern of maturation to the accumbens; again, overall binding decreased during puberty and again, this effect was more

pronounced in males than in females. This decline resulted in a main effect of age ($F_{1,17} = 10.11$, $p = 0.005$; Fig. 2B), and in a sex-by-age interaction ($F_{1,17} = 5.19$, $p = 0.03$). Bonferroni-corrected t-tests showed that the reduction in binding between juvenile and adult males was statistically significant: ($t = 3.94$, $p = 0.007$), and no such effect was present in females.

6.4.1.3 Piriform Cortex

In contrast to the tubercle and accumbens, the piriform cortex displayed a distinct pattern of maturation (Fig. 3A). Although male and female juveniles had identical binding levels, females' binding level increased during puberty while males' binding level decreased. This pattern of maturation resulted in a significant sex-by-age interaction ($F_{1, 17} = 19.55$, $p = 0.0004$; Fig. 3B), as well as a main effect of sex ($F_{1, 17} = 13.04$, $p = 0.002$). Bonferroni-corrected t-tests showed that binding levels changed significantly in both sexes (Males, $t = 3.7$, $p = 0.01$, Females, $t = 2.52$, $p = 0.004$).

6.4.1.4 Anterior cingulate cortex

CRF_1 binding in the ACC was very intense, clearly demarcating layer IV (Fig. 4A). Females had greater binding density than males overall, but binding decreased with age. Although the decline with age was greater in females, as shown by a Bonferonni corrected t-test ($t = 3.18$, $p = 0.04$), there was no interaction between sex and age. Nonetheless, there were main effects of sex ($F_{1,18} = 7.2$, $p = 0.01$) and of age ($F_{1,18} = 10.5$, $p = 0.004$; Fig. 4B).

6.4.1.5 Hippocampal formation, CA3 layer

CRF_1 binding in CA3 was quite weak relative to other examined areas (Fig. 5A). Nonetheless, across puberty, binding decreased in males and increased in females leading to a significant age-by-sex interaction ($F_{1,18} = 12.67$, $p = 0.002$; Fig. 5B). Males had significantly

greater binding than females before puberty ($t = 3.60$, $p = 0.0005$) however, binding decreased slightly in males and increased significantly in females, eliminating the sex difference in adulthood.

6.4.1.6 Hippocampal formation, dentate gyrus

CRF_1 binding in the DG was too faint to be reliably measured.

6.4.1.7 Paraventricular nucleus of the hypothalamus

CRF_1 binding in the PVN was too faint to be reliably measured.

6.4.2 CRF_2 binding

6.4.2.1 Lateral septum, intermediate subdivision

CRF_2 binding was more intense in the intermediate subdivision of the lateral septum (LSi) than the next most intense region, the VMH, by nearly two orders of magnitude. Ligand binding in the LSi formed an apostrophe-shaped region curving in a dorsomedial direction and terminating with its greatest intensity in the septohippocampal nucleus (Fig. 6A).

CRF_2 binding in the LSi was significantly greater in females than in males before puberty. However, binding decreased in females across puberty such that the pre-pubertal sex difference disappeared (Fig. 6B). This resulted in a significant interaction between sex and age ($F_{1,16} = 4.89$, $p = 0.04$).

6.4.2.2 Lateral septum, ventrolateral subdivision

In the ventrolateral subdivision of the LS, CRF_2 binding was approximately 50% less intense than in the intermediate subdivision. Females had greater binding than males but, in contrast to the LSi, they did not show a decline in binding during puberty. Consequently, females had greater binding than males regardless of age (Fig. 6B). This was reflected in a significant

main effect of sex ($F_{1,16}$ = 17.44, p = 0.0009), with no effect of age (p = 0.59), or interaction with sex and age. Bonferroni-corrected t-tests showed that the differences between males and females were statistically significant at both ages (both p's < 0.05).

6.4.2.3 Lateral septum, dorsal subdivision

In the dorsal subdivision of the LS (LSd; Fig. 6A), CRF_2 binding was half as intense as in the LSvl. Nonetheless, it showed a nearly identical pattern of maturation. Again, females had greater binding than males regardless of age. This was reflected in a significant main effect of sex ($F_{1,16}$ = 16.32, p = 0.0009; Fig. 6B), with no effect of age (p = 0.28), or interaction with sex and age.

6.4.2.4 Bed nucleus of the stria terminalis, principal subdivision

CRF_2 binding was very intense in the posterior BST (Fig. 7A) and clearly defined the rostral encapsulated, caudal columnar, and perifornical regions of the BSTpr. Binding in the BSTpr was greater in males than in females overall. Binding also increased across puberty to a slightly greater extent in males, leading to a significant sex difference at P98 (t = 2.93, p < 0.05; Fig. 7B). The overall pattern of results was reflected in a main effect of sex ($F_{1,20}$ = 13.4, p = 0.0001) and a strong trend toward an effect of age (p = 0.054).

6.4.2.5 Bed nucleus of the stria terminalis, interfascicular subdivision

CRF_2 binding in this region was roughly four-fold weaker than in the adjacent BSTpr (Fig. 7A). Nonetheless, the same basic pattern of results was obtained: Males had greater CRF_2 binding than females (Effect of sex, $F_{1,20}$ = 4.9, p = 0.03; Fig. 7B), and binding increased to a roughly equal extent in both sexes across puberty (Effect of age, $F_{1,20}$ = 4.6, p = 0.04).

6.4.2.6 Ventromedial hypothalamus, dorsomedial subdivision

Overall, CRF_2 binding in the VMHdm was among the most intense we have examined. Anatomically, binding density decreased in a continuous gradient along the main axis of the VMH, resulting in lower overall levels in the VMHvl (Fig. 5A) than in the VMHdm.

The VMHdm data were not normally distributed due to the spread among adult males, a few of which had exceptionally high optical density values. When the data were log-transformed to normalize variances across groups, ligand binding was found to increase across puberty (Fig. 5B). This resulted in a main effect of age in the VMHdm ($F_{1,19} = 8.27$, $p = 0.009$) with no effect of sex ($p = 0.2$), or interaction. However, CRF_2 ligand binding increased across puberty by an average of 7% in males and less than 1% in females, indicating that the effect of age was driven largely by the males. This impression was supported by t-tests, which showed that there was a statistically significant effect of age among males ($p = 0.01$) but not among females ($p = 0.35$).

6.4.2.7 Ventromedial hypothalamus, ventrolateral subdivision

A similar result to the VMHdm was obtained in the VMHvl: Ligand binding increased with age ($F_{1,19} = 7.1$, $p = 0.01$) and did not vary by sex ($p = 0.9$). However, the increase in binding across age was once again clearly greater in males than in females. This was supported by the trend toward an interaction between sex and age ($p = 0.07$) and by t-tests that compared juvenile and adult rats within each sex (males, $p = 0.01$; females, $p = 0.5$).

6.4.2.8 Paraventricular hypothalamic nucleus

The PVN had the lowest CRF_2 binding levels of any region examined. Unlike any other area, the characteristic shape of the PVN could not be discerned from the pattern of receptor binding. Binding levels were slightly greater in males than females overall, but this did not reach statistical significance ($p = 0.13$). The corrected binding levels (dpm/mg) for the PVN at P30

were males: 2.72 ± 0.27 and females: 1.54 ± 0.70. At P98 they were 2.48 ± 0.46 in males, and 1.77 ± 0.39 in females.

6.5 Discussion

The objective in this study was to test the hypothesis that the maturational pattern of CRF receptor binding elsewhere in the forebrain resembles that observed in the amygdala. The amygdala data were unambiguous: CRF_1 binding was greater in adult females in two of four areas and not dimorphic in the other two, whereas CRF_2 binding was greater in adult males in every examined region. Neither receptor subtype was dimorphic before puberty. As mentioned earlier, if CRF receptor maturation elsewhere in the brain resembled the amygdala, this would lend support to the idea that sex differences in binding levels could potentially explain sex differences in anxiety-like behavior. Thus, we measured type 1 and type 2 CRF receptor binding in several extra-amygdaloid brain areas.

The results indicate that our findings from the amygdala cannot be readily generalized to the rest of the brain. Although the general pattern of greater CRF_1 binding in females, and greater CRF_2 binding in males holds true for most of the areas we examined, CRF receptor binding is sexually dimorphic in some areas before puberty – unlike the amygdala. Thus, the main contrast between the four subdivisions of the amygdala we described earlier and the results we report here appears to be that these additional areas display greater variability in the timing of sexual differentiation. In the ACC, LS, and BST, CRF_1 or CRF_2 binding is greater in one sex before puberty, and it remains so in spite of whatever maturational changes are overlaid upon them. In contrast, CRF_1 binding in the AcbS and OT, and CRF_2 binding in the VMH, resembles the pattern in the amygdala: No sex difference in juvenile animals, followed by the emergence of a sex difference during pubertal maturation. The CRF receptor systems therefore demonstrate

several types of sexual dimorphism, making their functional significance a challenge to understand. Below, we review the findings from each brain region and discuss the potential mechanisms that could mediate these effects.

6.5.1 CRF_1 binding

In each of the five regions we measured, the maturation of CRF_1 binding presented a distinct pattern. For example, in the ACC, females had greater binding regardless of age. In the dorsal CA3, a sex difference that favored males was essentially reversed by a near-doubling in females and a slight reduction in males. In the basal forebrain of males, the AcbS and OT both demonstrated a massive reduction of binding, and a strong trend toward greater binding levels in adult females. Each of these patterns resulted in greater or near-greater binding levels in females. Thus, if one includes our findings from the amygdala in this discussion, the data indicate that females have greater CRF_1 binding levels overall than males.

Reviewing the behavioral role of all of the CRF_1-expressing brain regions examined in this study is beyond the scope of this discussion. Nonetheless, with the exception of the piriform cortex, each one has been implicated in anxiety, depression-like behavior, and/or regulation of the HPA axis. For example, the AcbS is well-known for its role in incentive motivation (Berridge et al., 2009), which is frequently decreased in major depression (Krishnan and Nestler, 2008; Nestler et al., 2002). The olfactory tubercle is considered part of the ventral striatum, a macrostructure that encompasses the accumbens and ventral pallidum (De Olmos and Heimer, 1999; Heimer et al., 1997), and thus also contributes to incentive motivation, particularly as it regards the relationship of olfactory cues to reproduction (Canteras et al., 1992; Cooper et al., 1994; Merchenthaler et al., 1984).

As described earlier, data from rodents and humans show that females are more prone to anxiety and depression than males, and are also more vulnerable than males to develop depression from adverse early experiences such as child abuse (Bourke and Neigh, 2011; Doom et al., 2013; MacMillan et al., 2001; MacMillan et al., 2009; Rao et al., 2008; Tarullo and Gunnar, 2006; Tolin and Foa, 2006; Weathington et al., 2012a). Moreover, considerable evidence suggests that CRF_1 activity is anxiogenic. If greater CRF_1 binding indicates more functional receptors and thus greater sensitivity to ligand, then the presence of greater or near-greater CRF_1 binding levels throughout the forebrain in females does lend credence to the notion that greater binding levels in females' could contribute to their greater vulnerability to mood disorders.

However, since neither the piriform cortex itself nor olfaction *per se* has been implicated in mood or anxiety, sexually dimorphic CRF_1 binding there suggests that the CRF_1 binding is, at best, incomplete. The piriform cortex is critical for the perception and memory of odors and can distinguish the odors of dominant and subordinate conspecifics (Veyrac et al., 2011). Males and females differ in their threshold to detect conspecific odors (Doty and Cameron, 2009; Sorwell et al., 2008), and gonadal hormones influence the impact of opposite sex olfactory cues in the piriform cortex (Kendrick et al., 1997). Moreover, estrogens reportedly influence CRF_1 expression in the piriform cortex (Foradori et al., 2007), suggesting that one way the F > M sex difference observed here could develop is through the effects of gonadal hormones directly on the piriform cortex, or indirectly, via its afferents.

Little is known about how CRF_1 influences synaptic transmission and information processing in regions such as the piriform cortex or AcbS. Data from the amygdala, however, indicate that CRF_1 enhances excitatory synaptic transmission and long-term potentiation (Fu et

al., 2007; Kash et al., 2008). In addition, the n-methyl-d-aspartate (NMDA) receptor and CRF_1 have a bidirectional relationship: NMDA receptor subunit phosphorylation influences CRF_1 expression (Delawary et al., 2010) and CRF_1 activity affects NMDA receptor channel conductance (Sheng et al., 2008). These findings are particularly interesting when considering that certain aspects of male reproductive behavior are incapable of being activated by androgen until after androgen exposure during puberty specifically (Sisk and Foster, 2004). It is conceivable that the maturation of CRF receptor expression, and its effects on synaptic transmission in regions such as the AcbS, piriform cortex, and amygdala, is what 'gates' the emergence of sex-typical behaviors during puberty.

It is interesting that the PVN, home of the principal CRF secretagogues of the anterior pituitary, should so weakly bind either CRF receptor. Maps of CRF_1 mRNA indicate low-to-moderate expression there (Chalmers et al., 1995; Van Pett et al., 2000). Nonetheless, CRF_1 binding was too faint to reliably visualize on the autoradiograms or to correlate with PVN cytoarchitecture in the Neutral Red stained tissue. Why there is detectable mRNA and low or no detectable binding is unclear at this point; it may be the technical limitations of autoradiography, or it may be that mRNA is not translated into protein. A third possibility is that there may actually be sex- or age-related differences in the surface-level *vs.* cytosolic distribution of CRF receptors, as suggested by the work of Bangasser et al. (2010).

6.5.2 CRF_2 binding

The dorsal and ventrolateral subdivisions of the lateral septum presented perhaps the most straightforward pattern of results, but one that was nonetheless unexpected: Irrespective of their age, females had *greater* CRF_2 binding than males and this remained unchanged across development. Females also had greater CRF_2 binding than males in the intermediate subdivision,

but as CRF_2 binding levels declined in females and remained relatively unchanged in males across puberty, this sex difference had disappeared by adulthood.

Greater CRF_2 binding in the LS of females is unexpected not only because of our previous results from the amygdala, but because Weiser et al. (2008) reported that the non-aromatizable androgen 5a-dihydrotestosterone (DHT) *increases* CRF_2 transcription and CRF_2 binding *in situ* in the LSvl and LSi. If androgen levels are positively related to CRF_2 mRNA expression in the LS, then one might have predicted that CRF_2 binding would be greater in adult males than in adult females, rather than the pattern observed here.

The pattern of CRF_2 binding in the posterior BST is more in line with what one might expect from a molecule that is up regulated by androgens. Males had greater binding levels before and after puberty. The pattern of CRF_2 binding in the posterior BST is thus clearly distinct from that of the amygdala because binding levels were sexually dimorphic before puberty and because females showed an increase in binding whereas they did not in the amygdala.

The dorsomedial and ventrolateral subdivisions of the VMH presented a distinct pattern of CRF_2 maturation from the LS and BST. Although both males and females both underwent an increase in binding during puberty, it is clear that the maturational effect was far greater in males than females, an impression reinforced with the results of post hoc tests. Thus, among all the regions examined, CRF_2 binding in the VMH most closely resembles the amygdala because the increase in binding occurred mainly in males.

6.5.2.1 Sexual differentiation of CRF receptors

The prevailing view of sex differentiation throughout the late 20th century is summarized by the genetic sex è gonadal sex è phenotypic sex model (Jost, 1971), which has successfully explained many neural sex differences. However, it is now clear that model was incomplete: In

addition to the effects of sex steroid hormones, there are a number of other parallel mechanisms that contribute to sexual differentiation. These include chromosomal sex differences and sex-typical experiences (reviewed in McCarthy and Arnold (2011)). It is necessary to systematically test each one in order to rule them out as factors that regulate CRF receptor binding. Until such studies are completed, we cannot identify what accounts for the sex-specific patterns of maturation we report here. Nonetheless, at the level of CRF receptors themselves, there are three non-mutually exclusive mechanisms that could explain these data: the number of receptors per neuron available for binding, changes in receptor binding affinity, and/or the number of CRF receptor-expressing neurons.

Sex differences in the availability of ligand could account for some or all of these data because G-protein coupled receptors are sequestered intracellularly upon ligand binding (Holmes et al., 2006; Jalink and Moolenaar, 2010; Oakley et al., 2007). Thus, there is presumably an inverse relationship between ligand availability and receptor binding. If sex differences in ligand availability underlie the patterns in CRF receptor binding, this begs the question as to how ligand availability becomes sexually dimorphic. Estrogen positively regulates *crf* transcription (Lunga and Herbert, 2004), which – if accompanied by greater CRF release – would *reduce* CRF_1 binding levels. Because we did not cycle the females in this study, we cannot rule out the possibility that estrous cycle phase contributed to the variance among the females observed here. However, the standard error term among the adult females in every area was similar to that of the prepubertal females, suggesting that estrous cycle-driven changes in ligand availability did not contribute significantly to the variance.

Another mechanism that could sexually differentiate CRF receptor binding is a change in the number of CRF receptors per cell. Since CRF_1 receptors are predominantly expressed on the

plasma membrane of dendritic shafts and spines (Jaferi et al., 2009), a change in dendritic branching could conceivably influence the number of binding sites. If neurons are growing or shrinking in a sex-specific manner between P30 and P98, then changes in the surface area available for receptor expression could account for the maturational effects observed here. The finding from Weiser et al. (2008) indicates that androgens directly regulate CRF_2 transcription, which could act independently of sex differences in morphological plasticity to influence the number of receptors per cell.

The coupling of G proteins with CRF receptors increases receptors' binding affinity (Ardati et al., 1999). Without knowing whether there is a sex difference in CRF_2 - G protein coupling, we cannot say whether differential binding affinity was a factor in the patterns described here. It should be noted, however, that Bangasser et al. (2010) reported a sex difference, favoring females, in CRF_1-G protein coupling in cerebral cortex homogenates of adult rats. If this finding pertains to the ACC, then the greater coupling of CRF_1 to G proteins in females would imply that receptor affinity is greater in females, and thus we would expect greater binding in females than in males, all else being equal.

Lastly, a change in the number of CRF receptor expressing neurons is another mechanism that could account for sex- and age-related effects on binding. Adult female rats reportedly have more apoptosis in the ACC than males (Sandau and Handa, 2006). If there were fewer neurons in females, this would reduce CRF_1 binding density in females' ACC. In the case of CRF_2s in the BSTpr, the number of receptor-expressing neurons may be greater in males because males have a greater number of BSTpr neurons than females before puberty (Forger et al., 2004; Guillamón et al., 1988). Our data could be explained by the presence of more CRF_2 -expressing neurons in prepubertal males, followed by a pubertal upregulation of surface-level expression in both sexes.

6.5.2.2 Understanding the function of sex differences in CRF receptor binding

The table shows that adult females have equal or greater CRF_1 binding levels than males, whereas adult males have more CRF_2 binding than females, the sole exception being the LS, where females have greater binding. As mentioned earlier, whereas the anxiogenic and HPA-activating effects of CRF_1 agonists are fairly clear, the role of CRF_2s in mood disorder-like behavior and HPA activity is less so. Nonetheless, if we provisionally accept the notion that CRF_1 activity is anxiogenic and has an HPA-activating effect and that CRF_2 activity can be anxiolytic in certain conditions and is involved in adapting the HPA axis to a long-term stressor, then these data are broadly consistent with adult females' higher levels of corticosterone (e.g., (Critchlow et al., 1963; Iwasaki-Sekino et al., 2009) and the greater tendency toward anxiety and depression among adult women that begins at puberty.

The F>M sex difference in the dorsal and ventrolateral LS was the only area in which adult females clearly deviated from the modal pattern for CRF_2s. What can this exception tell us? One possibility is that the sex difference exists to compensate for another sex difference, in order to decrease a difference between the sexes (De Vries, 2004). For example, the sexually dimorphic innervation of the LS by arginine vasopressin is exquisitely sensitive to circulating androgens and much greater in males than females. In the bi-parental prairie vole, gonadally intact males require LS vasopressin to display paternal behavior, whereas females do not (De Vries, 2004). An analogous situation could apply to the F>M sex difference in CRF_2 binding because urocortin3, a selective ligand for CRF_2, decreases the likelihood of maternal aggression when infused into the lateral septum (D'Anna and Gammie, 2009). Perhaps females' greater CRF_2 binding than males increases the probability of aggression, but after parturition receptor binding decreases, thus increasing its likelihood to equal that of males. Another, not mutually

exclusive possibility is that CRF_2s' effects are not unimodal; it is possible that CRF_2 activity in some regions is anxiogenic, anxiolytic, or neither. As noted earlier, this notion has precedence in studies of CRF_1 activity (Janssen and Kozicz, 2013). And indeed, Bakshi et al. (2007) report that blocking CRF_2s in the lateral septum has an anxiolytic effect, implying that LS receptors are normally anxiogenic. Thus, if greater CRF_2 binding implies greater agonist efficacy, then the greater number of CRF_2 receptors in females' LS would compound the pro-anxiety levels of CRF1 expression elsewhere in females' brain.

Our interest in CRF receptors was sparked by the sex-specific effects of juvenile social subjugation (JSS). Female adult rats subjected to JSS show more anxiety- and depression-like behaviors and greater HPA reactivity than adult males (Weathington et al., 2012a). Because nearly identical experiences yielded sexually dimorphic outcomes, we hypothesized that the neural circuits that mediate the experience of JSS are sexually dimorphic. Support for this idea came from sex-dependent, lateralized patterns of Fos immunoreactivity in response to JSS (Weathington et al., 2012b). Moreover, because we and others observed that social defeat-induced Fos co-localizes with CRF_2 immunoreactivity in the MeA (Fekete et al., 2009), this suggested that CRF_2 -expressing neurons in the MeA could be a substrate to mediate the sex-specific processing JSS. Therefore, it was somewhat unexpected that CRF_2 binding in the MeA was not sexually dimorphic in pre-pubertal rats. However, subsequent work in our lab has shown that the BSTpr is also activated by JSS in a sexually dimorphic manner (Weathington and Cooke). It remains to be seen whether JSS-induced Fos co-localizes with CRF_2-expressing neurons in the BSTpr, but this seems likely given how widespread and intense CRF_2 binding is in that region. Thus, given the anxiolytic effect of CRF_2s in the BSTpr in the context of

conditioned defeat (Cooper and Huhman, 2005), BSTpr CRF_2 receptors may be the crucial piece of the puzzle that explains why female rats are more susceptible to JSS than males.

6.6 Conclusions

In this paper, we have shown that the binding of type 1 and 2 CRF receptors undergoes a variety of changes during a 68-day span that encompasses the achievement of puberty and the passage through adolescence. In the wild, most female rats will normally have carried at least one litter through gestation to weaning by the time they are P98, and most male rats will have participated in the acts usually necessary to achieve reproductive success, including male-male aggression and sexual behavior (Calhoun, 1962). The CRF receptor system is clearly involved in adult social behavior, HPA axis regulation, and mood states such as anxiety, and sex differences have been identified in each of these functional domains. Presumably, the sex differences described here are adaptations that historically fostered male- and female-typical behaviors in ways that promoted reproductive success. Understanding what those ways are, however, requires first identifying the mechanism(s) that regulates CRF receptor binding, and then controlling it so as to sex-reverse its binding level in a site-specific manner, and observing subsequent effects on behavior.

6.7 Acknowledgments

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6.8 Chapter 6 Table

Table 2 Relative Sex Differences in CRFR binding

Receptor subtype	Brain region	Prepubertal (P30)	Postpubertal (P98)
CRF_1	AcbS	F ≈ M	F > M
	ACC	F > M	F > M
	BLA[a]	F ≈ M	F > M
	CA3	F ≈ M	F > M
	CeA[a]	F ≈ M	F ≈ M
	MePD[a]	F ≈ M	F ≈ M
	MePV[a]	F ≈ M	F > M
	OT	F ≈ M	F > M
	PIR	F ≈ M	F > M
CRF_2	BLA[a]	F ≈ M	F < M
	BSTif	F < M	F < M
	BSTpr	F < M	F < M
	CeA[a]	F ≈ M	F < M
	LSd	F > M	F > M
	LSi	F > M	F ≈ M
	LSvl	F > M	F > M
	MePD[a]	F ≈ M	F < M
	MePV[a]	F ≈ M	F < M
	PVN	F ≈ M	F ≈ M

VMHdm	F ≈ M	F < M
VMHvl	F ≈ M	F < M

[a] These data are summarized from Weathington and Cooke (2012).

6.9 Chapter 6 Figures

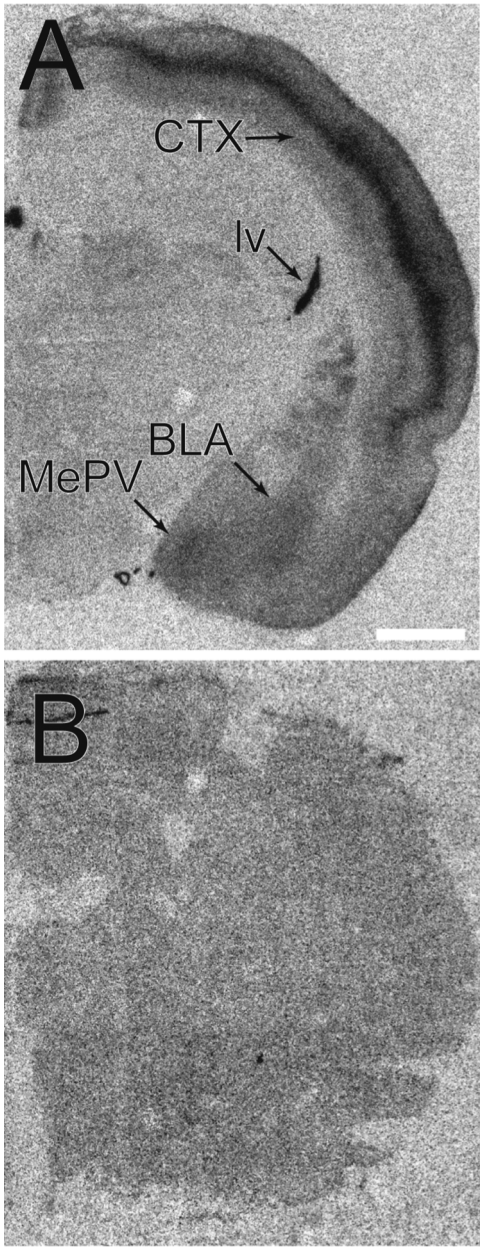

Figure 15 Selectivity of ^{125}I for CRF receptors

A. Section to which 0.2 nM ^{125}I sauvagine alone was applied. The presence of binding in regions that selectively express CRF_1 (cerebral cortex and basolateral amygdala) and CRF_2 (ventral medial amygdala) are indicated, indicating the ability of the ligand to bind to both receptors. **B.** Section to which both 500 mM CP-154,526 as well as 500 mM Astressin 2B, in addition to 0.2 nM ^{125}I sauvagine; the total absence of binding indicates the specificity of the antagonists. In this and in the remaining figures, the scale bar = 1 mm.

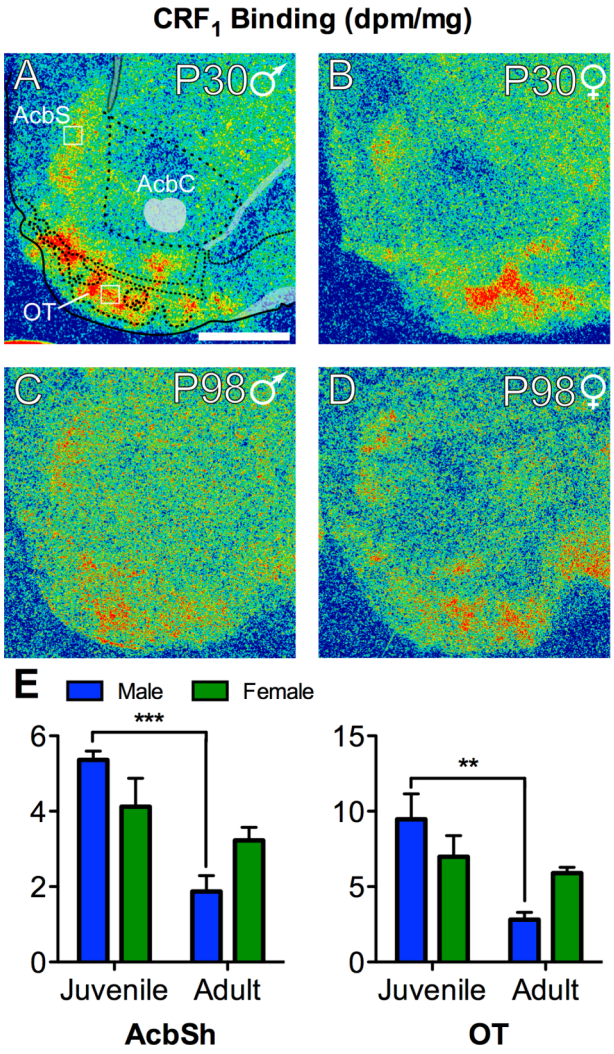

Figure 16 CRF_1 binding in the basal forebrain of male and female rats

A. CRF_1 binding in the basal forebrain of male and female rats. A - D. Heat maps of CRF_1 ligand binding in the OT and AcbS in prepubertal and adult male and female rats. Images represent rats whose values were closest to the mean of the AcbS. Boxes indicate the positions of the binding density measurements. **E.** Mean ± SEM optical density of CRF_1 -specific binding in the OT (bottom box) and AcbS. In both regions, males underwent a decline in binding during puberty, leading to significant sex-by-age interactions (AcbS: $F_{1,15} = 6.84$, $p = 0.01$; OT: $F_{1,17} = 5.19$, $p = 0.03$), as well as a main effect of age among males (t-tests, ** $p < 0.01$; *** $p < 0.001$).

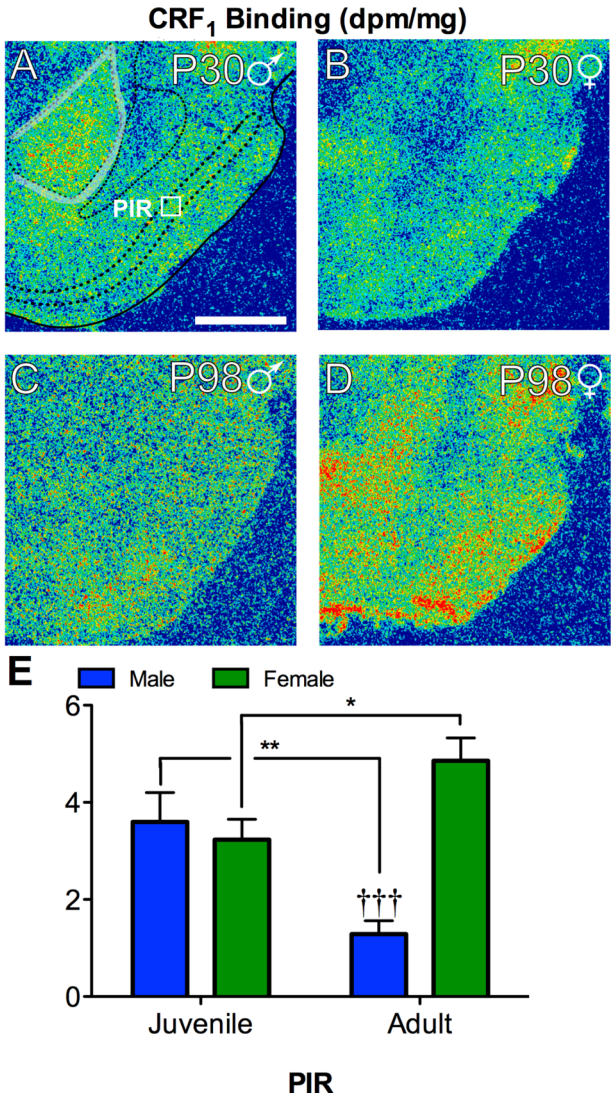

Figure 17 CRF_1 binding in the piriform cortex of male and female rats

A - D. Heat maps of CRF_1 ligand binding in the PIR of prepubertal and adult males and females whose binding density is closest to the mean of each group. Box indicates the position of the binding density measurement. **E.** Mean ± SEM optical density of CRF_1 -specific binding. Males' binding decreased across puberty, while females' increased, leading to a sex-by-age interaction (F1, 17 = 19.55, p = 0.0004; Fig. 3B), as well as a main effect of sex (F1, 17 = 13.04, p = 0.002). Binding levels changed significantly with age in both sexes (** p < 0.01). In addition, the sex difference in adulthood was highly significant (††† t = 5.8, p < 0.0001).

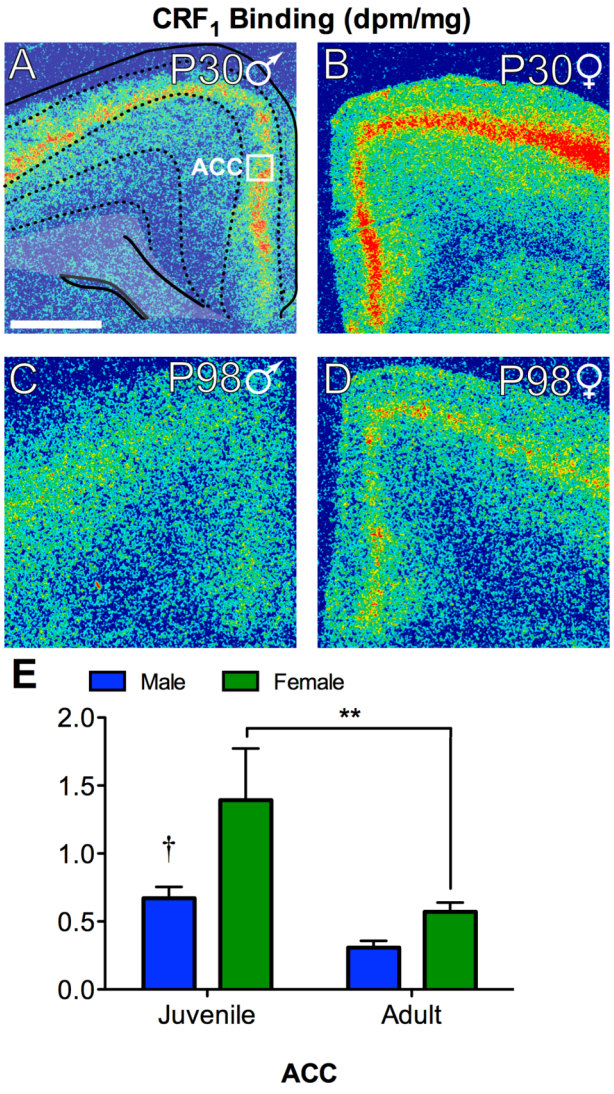

Figure 18 CRF_1 binding in the anterior cingulate cortex of male and female rats

A - D. Heat maps of CRF_1 ligand binding in the ACC of prepubertal and adult males and females whose binding density is closest to the mean of each group. Box indicates the position of the binding density measurement. **E.** Mean ± SEM optical density of CRF_1 -specific binding in the ACC. Binding was greater in females overall (effect of sex, $F_{1,18} = 7.2$, $p = 0.01$), with a greater effect size in prepubertal animals († $p < 0.05$) and both sexes showed a reduction in binding between P30 and P98 (effect of age, $F_{1,18} = 10.5$, $p = 0.004$). The reduction was statistically significant in females (**$p < 0.01$), not males.

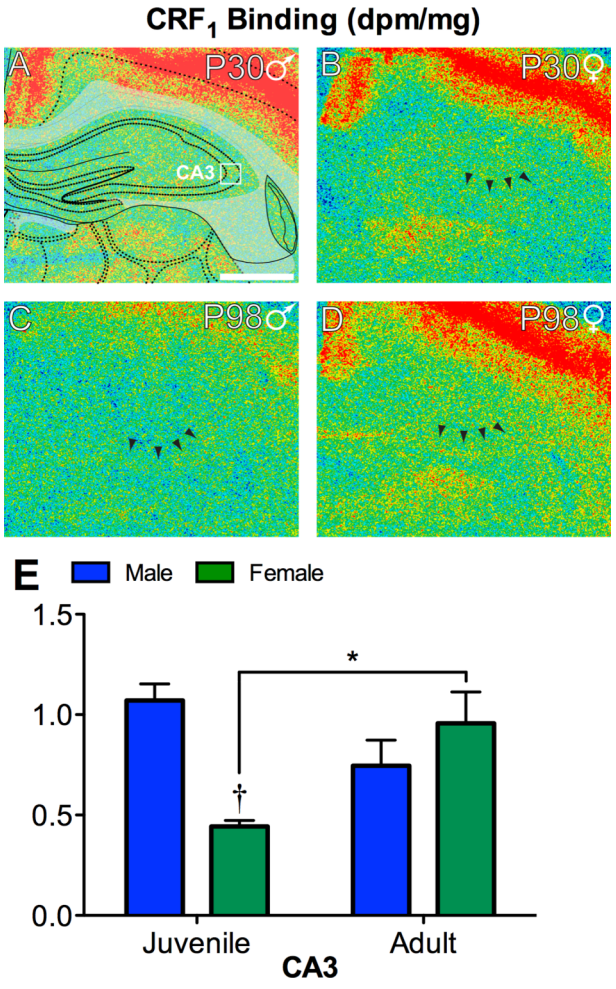

Figure 19 CRF_1 binding in the hippocampal formation of male and female rats

A - D. Heat maps of CRF_1 binding in the dorsal CA3 of four rats whose binding levels were closest to the mean of each group. The box indicates the position of the binding density

measurements. Because CRF_1 binding is so faint, arrowheads indicate its location in the other photomicrographs. **E.** A decrease in binding among males and an increase among females led to a significant sex-by-age interaction ($F_{1,18} = 12.67$, $p = 0.002$). Binding was significantly greater in prepubertal males († $p < 0.05$), but it increased across puberty in females (* $p < 0.05$), not in males.

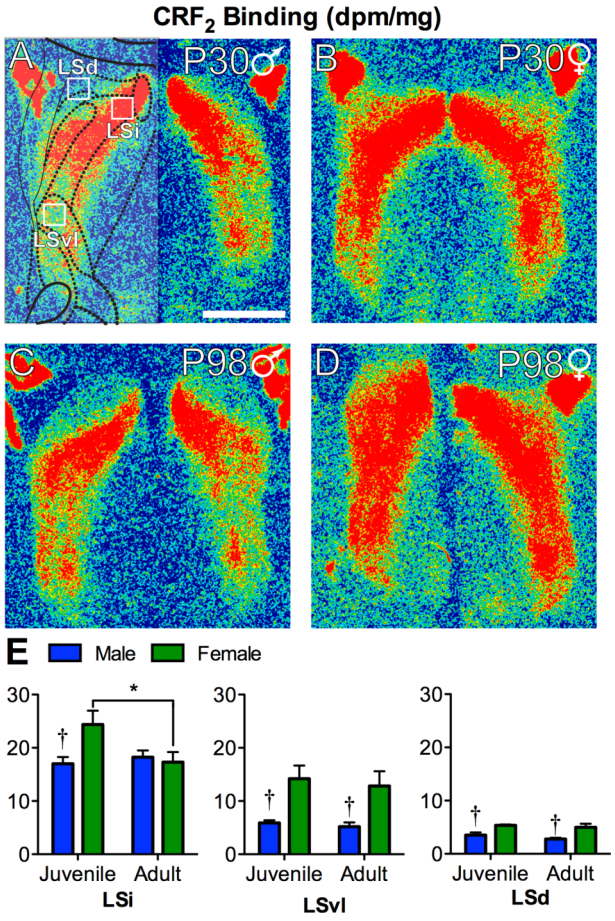

Figure 20 CRF_2 binding in the lateral septum of male and female rats

A - D. Heat maps of CRF_2 ligand binding in the lateral septum of prepubertal and adult males and females. Images are from rats whose values are closest to the mean for the LSd. Boxes indicate the positions of the binding density measurements. **E.** Mean ± SEM optical density of CRF_2 -specific binding in the intermediate (middle box), ventrolateral (bottom), and dorsal

subdivisions of the LS (Fig. 5B). Overall, females had significantly greater CRF_2 binding than males in every region (LSd, $F_{1, 15} = 35.3$, $p < 0.0001$; LSi, $F_{1,15} = 16.9$, $p = 0.0009$; LSvl, $F_{1,16} = 17.44$, $p = 0.0009$). t-tests showed that females had greater binding than males before puberty in the LSi, and at both ages in the LSd and LSvl († < 0.05), and that puberty significantly reduced binding in females' LSi (* < 0.05).

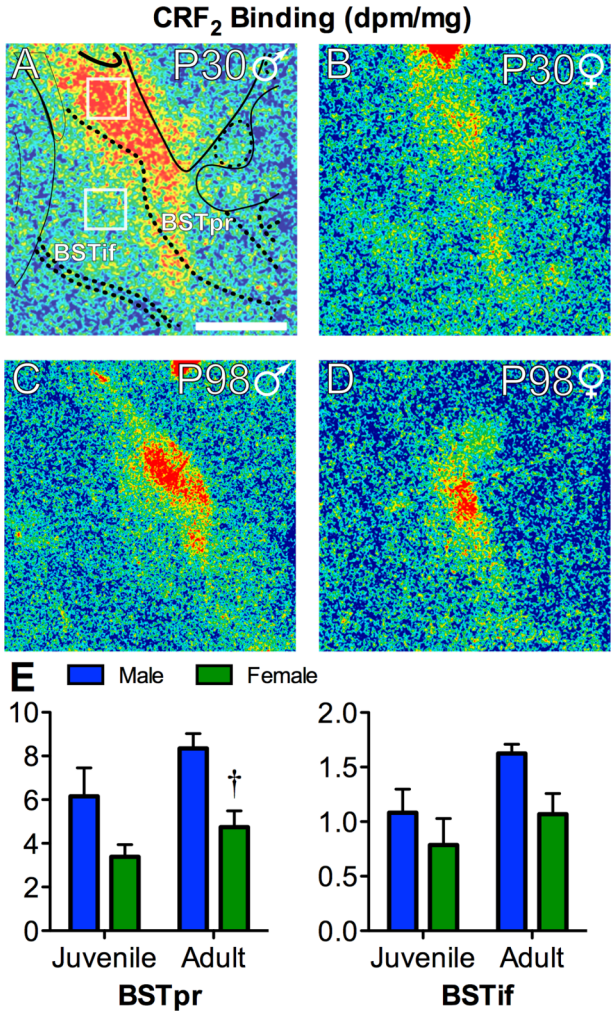

Figure 21 CRF_2 binding in the posterior BST of male and female rats

A - D. Heat maps of CRF_2 ligand binding in the posterior BST of representative prepubertal and adult males and females. Boxes indicate the positions of the binding density measurements. **E.** Mean ± SEM optical density of CRF_2 -specific binding in the principal (top box) and

interfascicular subdivisions of the posterior BST. In the principal subdivision, binding was greater in males overall (effect of sex, $F_{1,20} = 13.4$, $p = 0.0001$), and there was a trend toward an effect of age ($p = 0.059$). Males also had greater binding in the interfascicular subdivision (effect of sex, $F_{1,20} = 4.9$, $p = 0.03$) and there was a significant effect of age ($F_{1,20} = 4.6$, $p = 0.04$) in both sexes.

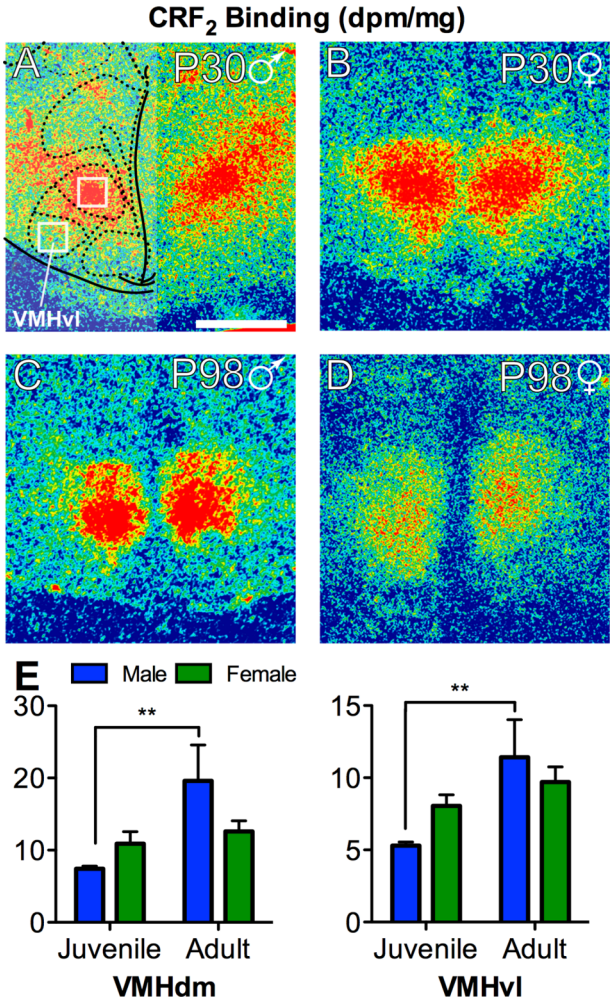

Figure 22 CRF_2 binding in the ventromedial hypothalamus of male and female rats

A – D. Heat maps of CRF_2 ligand binding in the VMH of four representative prepubertal and adult males and females. Boxes indicate the positions of the binding density measurements. **E.** Mean ± SEM optical density of $CRFR_2$-specific binding in the VMHdm (top box) and VMHvl.

There was a main effect of age in the VMHdm ($F_{1,19} = 8.27$, $p = 0.009$). Post hoc t-tests showed that this was largely due to the increase in binding among males ($p = 0.01$), not females ($p = 0.3$). A similar result was obtained in the VMHvl wherein binding overall increased with age (effect of age, $F_{1,19} = 7.1$, $p = 0.01$), but this was largely driven by the increase in males (** $p = 0.01$), not females ($p = 0.5$).

7 CHAPTER SEVEN: GENERAL DISCUSSION

7.1 Overview

Mood disorders are the most common stress-related psychiatric conditions, occurring with a lifetime prevalence of 15-20%, and incurring considerable social, occupational, and workplace costs (Kessler et al., 2005a; Wang et al., 2006). Furthermore, women develop these disorders twice as often as men, a sex difference that emerges during puberty (Angold and Costello, 2006; Nolen-Hoeksema, 1987). Early-life adversity such as physical abuse, sexual abuse, parental neglect, and bullying, is the most predictive environmental risk factor for anxiety and depression in adulthood (Edwards et al., 2003; Heim et al., 2000; Heim et al., 2008; Kaufman et al., 2000; Penza et al., 2003; Stapinski et al., 2015). In the US there are 500,000 documented cases of childhood physical or sexual abuse each year (Sedlak, 2010), and up to 10% of children report experiencing peer victimization (Juvonen et al., 2003; Nansel et al., 2001). Furthermore, maltreatment during early life is associated with a 2-fold increase in suicide attempts among adolescents and, a 5-fold increase among adults (Dube et al., 2001). Excessive or prolonged glucocorticoid exposure during development may impact stress responsive brain regions such that appropriate responding to future stressors is compromised, potentially leading to mood disorder susceptibility (de Kloet et al., 2005a). Despite equal rates of abuse among boys and girls in the U.S., women maltreated during childhood are more likely to develop stress-related mood disorders in adulthood than males (Altemus, 2006; Becker et al., 2007; CWLA, 2006; Heim et al., 2008; Heim et al., 2002; Lenze et al., 2008; Pine et al., 2002; Tarullo and Gunnar, 2006). Current research suggests that females may be disproportionally susceptible to the negative effects of early-life stress, predisposing those with a history of early adversity to stress-related pathologies later in life (Becker et al., 2007; De Bellis et al., 1999a; Kendler et al.,

1999). Importantly, the developmental origins and neural mechanisms underlying female-biased mood disorder prevalence remain largely unknown.

Human studies are limited in their capacity to explore causal relationships between early-life adversity and adult psychopathology as these studies typically rely on correlations between adult participants' retroactive recollection of maltreatment experiences and current symptoms (Breslau, 2002). Furthermore, clinical studies are unable to control the type, frequency or magnitude of early adversity, or pinpoint neural mechanisms by which abuse and maltreatment affect the brain. By addressing some of these limitations, rodent studies have demonstrated many long-term effects of chronic early life stress exposure. Surprisingly, few animal studies have investigated sex-specific effects of adolescent social stress on adult behavioral and neuroendocrine pathologies associated with mood disorders. Likewise, investigations into sex-specific processing of acute social stress, or maturational differences in the expression of stress-responsive neural substrates, are lacking among animal studies. Overall, the studies of this dissertation aimed to address these gaps. Specifically, we investigated adult mood disorder-like behavioral and neuroendocrine consequences of chronic Juvenile Social Subjugation (JSS), a social defeat-related rodent model of adolescent social stress that includes female subjects. We also examined sex-specific patterns of neuronal activation following acute JSS exposure and maturational differences in the CRF receptor system.

7.2 Experimental Findings: Potential Mechanisms and Implications

7.2.1 Chronic JSS induces sex-specific mood disorder-like behavior in adulthood

Animal models of chronic adolescent stress including chronic variable stress, predator odor, social instability, and social defeat, have been shown to induce mood-disorder like behaviors that persist into adulthood (for review see (Holder and Blaustein, 2014)). Although the

majority of these studies involve only male subjects, similar behavioral outcomes are observed in females (Bourke and Neigh, 2011; McCormick et al., 2008; Pohl et al., 2007). It has been suggested that animal models of social stress that occur within an evolutionarily meaningful social context offer the most face validity; thus, social defeat is more germane than nonsocial or physical stressors when studying mood disorder-related outcomes (Schmidt et al., 2010; Tsankova et al., 2006). However, it is difficult to include female subjects in animal social defeat models because they do not exhibit male-typical aggression, territoriality, or hierarchy formation (Donner and Lowry, 2013; Palanza et al., 2001; Razafsha et al., 2013).

Our behavioral studies, discussed in chapter 2, found that daily exposure of male and female juvenile rats to an aggressive adult male induced stress-related mood disorder-like behavior in adulthood, and these effects were predominantly observed in females. Specifically, chronic JSS increased anxiety-like behavior on the EPM, increased depression-like behavior on the FST, and reduced sociability on the SIT in females, and these changes lasted into adulthood (Weathington et al., 2012a). Although few studies have investigated effects of chronic social stress in adolescent male and female subjects, the majority of previous findings are in line with ours. These studies observe sex-specific and long-lasting alterations in anxiety- and depression-like behavior and sociability after social instability stress (Mccormick et al., 2005; McCormick et al., 2008; Pohl et al., 2007), as well as social defeat stress (Bourke and Neigh, 2011), with the exception of two reports (Mathews et al., 2008; Ver Hoeve et al., 2013). For example, daily social isolation followed by cage partner change induces adult anxiety-like behavior on the EPM in females, but not males (McCormick et al., 2008). Similarly, chronic variable stress increases depression-like behavior in the sucrose preference exclusively in females (Pohl et al., 2007). This is also true of one report that used male and female adult residents to achieve same-sex social

subjugation, where perhaps due to strain differences, adequate territorial aggression of adult female aggressors was induced by ovariectomy (Bourke and Neigh, 2011). In this study, a mixed-modality paradigm consisting of adolescent social defeat, social isolation, and restraint stress induced depression-like behavior in the sucrose preference test and reactive stress coping (immobility) in the FST in females but not males, in adulthood (Bourke and Neigh, 2011). Another report utilizing adult female lactating rats as resident aggressors observed transient depression-like behavior after social defeat in juvenile females but this did not persist into adulthood (Ver Hoeve et al., 2013).

7.2.2 Chronic JSS induces neuroendocrine dysfunction in adulthood

In addition to alterations in mood disorder-like behavior, the majority of animal adolescent social stress models also induce long–lasting HPA axis dysfunction (Bourke and Neigh, 2011; McCormick et al., 2008; Pohl et al., 2007). However, it has been pointed out that, of these models, those with earlier onset of stress tend to report altered HPA function more often (Holder and Blaustein, 2014). Also, models of social stress generally cause more frequent and longer-lasting HPA alterations in female subjects than do nonsocial stressors (McCormick and Green, 2013). In our studies, JSS-exposed females showed higher stress-evoked CORT levels and heavier adrenal glands in adulthood than control females, or males (Weathington et al., 2012a). Likewise, elevated stress-evoked CORT was seen in adult females, but not males, subjected to adolescent social instability (Pohl et al., 2007). On the other hand, two studies observed reduced basal ACTH and slower hormonal stress recovery (Ver Hoeve et al., 2013), as well as reduced stress-evoked CORT (Bourke and Neigh, 2011) in adult females that were socially defeated during adolescence by female adult residents.

Clinical studies generally report an association of childhood maltreatment and mood disorder pathology with HPA axis dysregulation, with some patients exhibiting increased, and others showing decreased basal and evoked stress hormone levels. For example, depressed women with a history of childhood maltreatment show greater ACTH and cortisol responses following a laboratory psychosocial stressor than non-depressed women that experienced childhood maltreatment, or depressed women without such history (Heim et al., 2000). A similar study by the same group revealed a blunting of ACTH responsivity to intravenous CRF challenge in depressed women, with or without a history of abuse, compared to non-depressed abused women (Heim et al., 2001). Discrepancies in the directionality of stress hormone alterations in these studies has been suggested to reflect current patient stress levels, interaction effects of treatment, and state-dependent changes, which involve improving HPA axis changes as depression symptoms subside (Heim et al., 2001).

Although neuroendocrine dysfunction and affective disorder co-occurrence is a well-established psychiatric finding, only about 50% of depressed patients reliably exhibit aberrant HPA axis activity (Carroll, 1982). Clues as to why affective disorders inconsistently induce hyper or hypocortisolism remain elusive. It has been suggested that imbalanced GR and MR signaling could be involved, as they typically induce strong modulatory effects on the direction and magnitude of HPA axis activity (Baes et al., 2014). Indeed, the brains of depressed patients present reduced GR and MR transcript levels in areas including the anterior cingulate cortex, dorsolateral PFC, inferior frontal gyrus, hippocampus, and amygdala (Alt et al., 2010; Klok et al., 2011; Medina et al., 2013; Perlman et al., 2004; Qi et al., 2013; Wang et al., 2014).

7.2.3 Sexually dimorphic neural processing of juvenile social stress

Sex-specific neural processing of adolescent stress may provide a mechanism underlying sex differences in the effects of early life stress exposure. To test the hypothesis that JSS elicits disparate patterns of neural activation in males and females, we examined immunoreactivity (ir) of Fos, an immediate early gene and widely used proxy for neuronal activity, in stress-responsive brain regions after either exposure to a single episode of JSS, social encounter with a benign adult male rat (BC), or handling. The addition of the benign control condition aimed to control for social experience as the intruders are housed individually beginning at weaning. While the behavioral studies involved chronic JSS exposure, Fos-ir was examined after acute JSS exposure. Overall, our immediate early gene studies, detailed in chapters 3 and 4, report sex-, region-, and experience-specific neuronal activation patterns following JSS, BC, or handling experiences (Weathington et al., 2013; Weathington et al., 2012b). Generally, Fos-ir was highest in the PVN and MeA, intermediate in the BLA, and low in the CeA and BSTpr. While few reports have examined these measures, our results are consistent with Fos-ir after acute social defeat in males (Boguszewski and Zagrodzka, 2005; Fekete et al., 2009; Martinez et al., 2002).

CeA neuronal activation was very low overall, and did not differ among experimental conditions. This was expected as the MeA preferentially responds to psychogenic while the CeA is selective for physical stressors (Dayas et al., 1999; Dayas and Day, 2002). The CeA is the amygdala's major output, projecting to the brainstem and hypothalamus (Pitkänen et al., 1997), and low neural activation could reflect its high GABA receptor abundance and thus inhibition (Parisa et al., 2008). In both males and females, MePV neuronal activation increased following JSS and BC experiences, while MePD neuronal activation increased only after JSS, suggesting a more general responsiveness of the MePV to social stimuli, while the MePD may be selectively sensitive to characteristic features of JSS. The MePV plays roles in threat detection as well as

defensive behaviors such as fear (Canteras, 2002; Dielenberg and McGregor, 2001) suggesting similar threat levels in juvenile male and females. Gonadal hormone receptors are intensely expressed in the MePD (Simerly et al., 1990; Wood et al., 1992), which is larger in adult, (Cooke et al., 1999; Hines et al., 1992) and juvenile males, perhaps tuning the MePD response to social stressors such as JSS. Interestingly, BSTpr neural activation reflected that of the MeA, perhaps owing to their direct interconnectivity (Canteras et al., 1995; Cooke and Simerly, 2005; Dong and Swanson, 2004). In humans, a history of childhood maltreatment is associated with amygdala hyperreactivity among anxious and depressed patients (Teicher and Samson, 2013a). Furthermore, individuals with PTSD or anxiety show increased amygdala activation in response to threatening faces (Kim et al., 2011; Shin et al., 2005; Stein et al., 2007).

JSS increased neural activation in the posterodorsal MeA (MePD) of males. The same response was observed in the left MePD of females, while the right MePD responded intermediately to all three conditions. Hemispheric lateralization of neural processing could contribute to sex-specific effects as lateralized synaptic morphology is reported in the male MePD (Cooke et al., 2007b; Cooke and Woolley, 2005b). In humans, emotional stimuli induce lateralized amygdala activation in both sexes (Armony and Sergerie, 2007; Cahill et al., 2001; Stevens and Hamann, 2012). For example, the right hemisphere is reported to be more involved in processing negative affect than the left (Otto et al., 1987).

Fos-ir was highest in the PVN, where males showed more robust Fos-ir after JSS and handling than BC, suggesting potential social buffering by benign social experience. It is possible that male intruders and benign residents engaged in play behavior, which is reported to promote coping with social changes (van den Berg et al., 1999). In humans, social support is known to mitigate psychiatric disorder risk (Brown et al., 1994). Neuronal activation in the BLA

was observed after JSS and BC in females, but only after JSS in males, suggestive of sex-specific BLA-mediated emotional arousal.

7.2.4 Development of sex differences in the CRF system

To investigate stress-related vulnerabilities of biological sex and age we used competitive ligand receptor binding autoradiography to selectively label CRF1 and CRF2 receptors in male and female, adolescent and adult rats. We quantified CRF receptor binding in stress-related forebrain regions including the anterior cingulate cortex (ACC), layer CA3 of the hippocampus (CA3), subregions of the lateral septum (LSi, LSvl, LSd), BSTpr, and subregions of the amygdala (BLA, CeA, MePD, and MEPV). Overall, our autoradiography studies, detailed in chapters 5 and 6, report sex-, age-, and region-specific expression of CRF1 and CRF2 receptors (Weathington and Cooke, 2012; Weathington et al., 2014). Overall distributions and gonadal hormonal effects on CRF receptor mRNA and protein in the adult rat brain have previously been described and are consistent with our results (Avishai-Eliner et al., 1996; Chalmers et al., 1995; Reul and Holsboer, 2002).

Prepubertal sex differences in CRF1 binding the ACC and CA3 could contribute to sex-specific stress processing during adolescence, as these differences disappeared in adulthood. Adolescent females show greater CRF1 binding in the ACC, suggesting that they may attribute higher emotional valence to sensory stimuli than males. Conversely, adolescent females show lower CRF1 binding in the CA3, suggesting lower levels of HPA axis feedback than males. In the amygdala, CRF1 binding was indistinguishable between adolescent males and females in each subregion examined. In adulthood, females display greater CRF1 binding in the BLA and MePV than males. Due to the clear role of CRF in the initiation of the HPA axis, autonomic, and behavioral responses to stress, higher CRF1 binding in regions of the amygdala known to

modulate emotion and threat detection (Jessica et al., 2013b; Pitkänen et al., 1997) suggests greater sensitivity of adult females to stressors relative to males.

Although adolescent males and females show comparable levels of CRF2 binding in the amygdala, male CRF2 binding increased across puberty in all subnuclei examined. Females displayed higher overall CRF2 binding among LS subnuclei than males. Recently, CRF2 receptors in the LS were confirmed to increase anxiety-like behavior (Todd et al., 2014), and therefore higher LS CRF2 in females may contribute to higher anxiety-like behavior after chronic stress exposure. On the other hand, males displayed greater overall CRF2 binding among BSTpr subnuclei than females; CRF2 receptors in the BSTpr decrease sustained fear after stress in adult rats (Elharrar et al., 2013).

Combined results suggest that males tend to have greater CRF2 binding, while females tend to have greater CRF1 binding in adulthood. Developmental increases in female CRF1 binding support high adult female stress sensitivity. Estrogens are known to upregulate CRF expression (Lunga and Herbert, 2004), so pubertal increases in ligand availability may mediate widespread developmental increases in CRF1 expression in females. Likewise, testosterone upregulates CRF2 mRNA (Weiser et al., 2008), and overall developmental increases in CRF2 binding in males may be mediated by pubertal hormones.

7.3 Bi-directional Interactions of Sex and Stress

Both stress effects and sex differences involve steroid hormone action in the brain. Stress hormones are logical candidates underlying environmental risk factors such as early life stress. Similarly, gonadal hormones likely mediate risk factors associated with biological sex. In addition, there are several potential points of crosstalk between sex and stress (i.e. sex hormones affecting the stress response, or conversely, stress affecting gonadal hormone effects). Steroid

hormones function intracellularly as transcription factors, and response elements for both androgens and estrogens (AREs and EREs) are located on the upstream promoter of the CRF2 receptor gene (Catalano et al., 2003). These AREs and EREs could be sites where changes in local or circulating hormones directly influence CRF2 expression and activity and, hence, the response to a stressor.

Conversely, stress can indirectly alter gonadal hormone effects on the brain. For example, stress during adolescence can affect pubertal onset, which in turn alters the timing of gonadal hormone exposure in the adolescent brain (Kinsey-Jones et al., 2010). Pubertal onset may also be programed by gestational or neonatal glucocorticoid exposure (Harvey and Chevins, 1987a, b; Herrenkohl, 1979; Smith and Waddell, 2000). In humans, premature pubertal onset is an established risk factor for the development of anxiety and depression among adolescent females (Ge et al., 2001; Kaltiala-Heino et al., 2003; Stice et al., 2001). Interestingly, neurons within the anteroventral periventricular nucleus (AVPV) that initiate gonadotropin-induced pubertal onset express CRF1 receptors (Takumi et al., 2012). During adolescent stress, CRF may act via CRF1 receptors on AVPV neurons to alter pubertal timing, and thus change circulating gonadal hormones levels, which affect mood (Steiner, 2003).

7.3.1 Epigenetics may mediate effects of sex and stress

Epigenetic mechanisms are particularly well suited to underlie experience-dependent changes in the brain and behavior. The most well known epigenetic mechanisms involve the chemical modification of chromatin, histones and DNA, and altering gene expression. Several families of enzymes are responsible for epigenetic modifications: DNA methyl-transferases (DNMTs) add methyl groups to DNA, and histone transferases (HATs) add acetyl groups to histones while histone deacetylases (HDACS) remove them. Studies have observed sex and

stress hormone-specific epigenetic changes in the brain. For example, patterns of global DNA methylation in the BNNT/POA mice were recently found to be sexually dimorphic and organized by perinatal testosterone (Ghahramani et al., 2014). Sex-specific patterns of CRF promoter methylation were observed in rats after exposure to chronic mild variable stress; specifically, methylation decreased in the BNST of males and females, and increased in the PVN of females only (Sterrenburg et al., 2012). Low maternal care in rats decreased the specific histone acetylation mark, H3K9, and increased methylation of the GR promoter in the hippocampus (Weaver et al., 2004). While sex and stress hormones are able to alter epigenetic marks, exactly how this happens is still not clear. Steroid hormones may affect expression of enzymes that cause epigenetic changes such as DNMTs, HATs, or HDACs, or may recruit chromatin-modifying proteins to genes. A speculated epigenetic consequence of early adversity is the stress hormone-modulated alteration of key genes, or the ability of key genes to be expressed in response to future stressors, which may predispose individuals to mood disorder development (Bagot et al., 2014). Although the field of neuro-epigenetics is still nascent, detailed knowledge of epigenetic mechanisms underlying environmental predisposition to mood disorder development are likely to advance in the future.

7.4 Future Directions for Stress-Related Mood Disorder Treatment: CRF Pharmacotherapy

Currently, the first line of treatment for depression is the use of selective serotonin reuptake inhibitors. Unfortunately, these drugs commonly induce side effects, and 30 to 50% of depressed patients do not respond to treatment (Juruena et al., 2015). Furthermore, patients with a history of childhood maltreatment are more likely to be diagnosed with treatment resistant depression (Kaplan and Klinetob, 2000; Teicher and Samson, 2013b). Because of the limited efficacy of mood disorder drug treatment, many researchers are trying to better understand the

etiology and physiopathology of depression, while looking for biological and psychosocial factors that predict treatment outcome (Costello et al., 2002; Emslie et al., 2003; Tamminga et al., 2002).

Rodent studies have established the role of the CRF1 receptors in initiating neuroendocrine, autonomic, and behavioral responses to stress (Laryea et al., 2012). In rodents, chronic antidepressant treatment reduces CRF transcripts in the PVN and HPA axis activity in response to acute stress (Stout et al., 2000). Thus, the successful treatment of major depression may involve the normalization of HPA hyperactivity by pharmacological intervention of the CRF system (Binder and Nemeroff, 2010). Toward this end, clinical studies involving CRF1 antagonists have been conducted. While some clinically tested CRF1 antagonists were unsuccessful due to side effects or toxicity concerns, others have been promising. In particular, the CRF1 antagonist R121919 has been shown to reduce depression and anxiety scores (Holsboer and Ising, 2008). Recently, another CRF1 antagonist, R317573, has been shown to decrease subjective symptoms in a human model of anxiety (Bailey et al., 2011). Currently, a clinical study is underway to test the treatment efficacy of another CRF1 antagonist, GSK561679, with post traumatic stress disorder specifically in women (Dunlop et al., 2014).

7.5 Concluding Remarks

Like most psychiatric disorders, the development of mood disorders involves multiple risk factors. Numerous preclinical and clinical studies have shown that exposure to abuse or maltreatment during early life increases susceptibility to the development of stress-related mood disorders, and females may be especially sensitive to long-lasting, negative effects of early-life stress. However, neural mechanisms underlying female-biased mood disorder prevalence remain largely unidentified. Collectively, this dissertation contributed to understanding the behavioral

and brain mechanisms whereby stressful early-life experience may interact with biological sex to induce sex-specific vulnerabilities to stress-related psychiatric disorder development.

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