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Vasiliki Michopoulos **Date**

Psychosocial stress induces distinct physiological and behavioral phenotypes in female rhesus monkeys

By

Vasiliki Michopoulos

B.A, University of Virginia, 2005

Neuroscience

Mark E. Wilson, PhD
Advisor

Becky Kinkead, PhD
Committee Member

Kerry J. Ressler, MD/PhD
Committee Member

Mar Sanchez, PhD
Committee Member

Kim Wallen, PhD
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

Date

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Abstract

Psychosocial stress induces distinct physiological and behavioral phenotypes in female rhesus monkeys

By Vasiliki Michopoulos

Exposure to chronic stressors is a causal and sustaining factor in a number of adverse health outcomes, an observation supported by an extensive literature from epidemiological analyses in humans. Dysfunction of the limbic-hypothalamic pituitary-adrenal (LHPA) axis is characteristic of psychopathologies and other stress-induced disease states that are highly co-morbid, including cardiovascular disease, immune dysfunction, reproductive compromise, emotional feeding and obesity. We propose in this dissertation that psychosocial stress exposure in the form of social subordination results in stress-induced alterations in behavior and physiology in female rhesus monkeys. In a series of experiments, we show that social subordination in female rhesus monkeys dysregulates LHPA activity, decreases sensitivity to the socioemotional effects of estradiol, alters feeding behavior in complex dietary environments, and disrupts reproductive function. This dissertation work provides evidence that social subordination results in two distinct phenotypes that can be studied to assess the etiology of stress-induced disruptions in behavior and physiology due to psychosocial stress exposure specifically in females.

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CHAPTER ONE:

ADVERSE HEALTH CONSEQUENCES DUE TO EXPOSURE TO CHRONIC STRESSORS IN HUMANS: IMPORTANCE OF DEVELOPING A TRANSLATIONAL ANIMAL MODEL

1.1 Abstract

Exposure to chronic stressors is a causal and sustaining factor in a number of adverse health outcomes, an observation supported by an extensive literature from epidemiological analyses in humans. Dysfunction of the neuroendocrine control of the limbic-hypothalamic pituitary-adrenal (LHPA) axis due to continuous exposure to stressors has lead to an increase in the prevalence of psychopathologies and other disease states that are highly co-morbid, including cardiovascular disease, immune dysfunction, reproductive compromise, emotional feeding and obesity. The health burden associated with disorders whose etiology is linked to chronic stress exposure necessitates the development of appropriate animal models with which to study the etiology of stress-induced changes in behavior and physiology in a relevant translational manner. Furthermore, women suffer from these disorders at a rate two to one over men, requiring the development of an animal model to address stress-induced alterations in behavior and physiology specifically in females. Here we discuss the neuroendocrine control of stress, and animal models currently employed to study the etiology of stress-induced changes in behavior and physiology. Finally, we propose that studying social subordination in female rhesus monkeys is a valid animal model of psychosocial stress exposure with which to study stress-induced alterations in behavior and physiology in females because subordinate females are subjected to daily harassment that results in adverse health consequences due to their social status. We hypothesize in this dissertation that social subordination dysregulates LHPA activity, and produces distinct behavioral, metabolic and reproductive phenotypes between dominant and subordinate females.

1.2 Stress in modern society and its ramifications on human health

A stressor is any perceived threat that jeopardizes an organism's well being and induces physiological changes that activate systems crucial for surviving the perceived threatening stimulus and restoring homeostasis (Chrousos and Gold, 1992; McEwen, 1998). Stressors can be physical or psychological in nature, can be discrete events or continuous assaults on homeostasis, and can have both physiological and behavioral consequences for an organism (Marmot, 2006). A stress response is the rapid activation of the sympathetic nervous system and the limbic-hypothalamic pituitary-adrenal (LHPA) axis in response to a threat and likely evolved to promote survival from acute physical stressors (Chrousos and Gold, 1992). However, stressor exposure in modern day human societies increasingly takes the form of psychological stressors that are experienced on a daily basis (Marmot, 2006). Continuous activation of these neuroendocrine pathways under such conditions is deleterious to one's health as evidenced by the increase of disorders whose etiology stems from chronic exposure to stressors (Marmot, 2006; McEwen, 1998).

Indeed, exposure to chronic stressors is a precipitating factor in a number of adverse health outcomes (Juster et al., 2010; McEwen and Gianaros, 2010), an observation supported by an extensive literature from both prospective animal studies and epidemiological analyses in humans. One class of disorders whose increase in prevalence is significantly noted over the last 40 years is psychiatric disorders, including depression, anxiety and posttraumatic stress disorder (Brundtland, 2001). As of 2001, the World Health Organization (WHO) reported that 450 million people worldwide suffer from psychopathologies, with an estimated one in four individuals suffering from a

psychopathology during their lifetime (Brundtland, 2001). By year 2020, the number of individuals suffering from psychopathologies will be only second to those ailing from cardiovascular disease (Brundtland, 2001). Already, the estimated health burden for depression alone worldwide is ranked fourth overall with depression being one of the leading causes of disability in modern society (Brundtland, 2001). The etiology of stress-induced psychopathologies, and the behavioral alterations and symptoms associated with them, stem from a dysregulation, or altered regulation, in the function and control of LHPA axis (Chrousos and Kino, 2007; Holsboer, 2001; Raison and Miller, 2003).

Chronic exposure to stressors and heightened neuroendocrine stress responses are also implicated in the etiology of other adverse health outcomes beyond psychological disorders, including immune dysfunction induced by chronic activation of the innate immune response and increased inflammation (Miller, 2010). Increased exposure to continuous stressors also augments individual vulnerability to cardiovascular disease in humans (Brunner, 1997; Marmot, 2006) and non-human primates (Kaplan et al., 2009; Shively et al., 1990a). Additionally, disruptions in the function of the reproductive axis due to chronic stress exposure lead to hypothalamic hypogonadism and infertility in both men (Wainwright et al., 2011) and women with functional hypothalamic amenorrhea (Berga and Girton, 1989). Stressful experiences also increase individual susceptibility to addiction (Koob and Kreek, 2007), such as drug abuse (Sinha, 2008) and alcoholism (Oroszi and Goldman, 2004), as well as emotional feeding (Greeno and Wing, 1994) and obesity (Bjorntorp, 2001; Rosmond, 2004; Scott et al., 2008). Because these stress-induced adverse health effects do not occur in isolation, but are often co-morbid, understanding how chronic psychosocial stress exposure disrupts multiple physiological

and behavioral systems concurrently is critical to developing efficacious and effective interventions with which to treat individuals suffering from stress-induced diseases.

1.3 Neuroendocrine regulation of stress reactivity

While a physical stressor that results directly from infection or injury can initiate a similar cascade of biological changes, the psychogenic component of exposure to social stressors is important and involves activation of cortico-limbic circuits that modulate both sympathetic and LHPA responses (Choi et al., 2008; Herman et al., 2003; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). In general, the specific parameters of the stress response can vary depending on whether the socio-environmental stressor is acute (short duration), chronic (prolonged duration), or acute imposed on the background of chronic stress. Whereas acute stress activates sympathetic and hormonal events which orchestrate a coordinated sequence of responses to restore homeostasis or allostasis, as described by McEwen and colleagues (McEwen and Wingfield, 2010; Schulkin et al., 1994), chronic stress can overwhelm these allostatic mechanisms and results in dysregulation or mal-adaptations of central and peripheral circuits regulating the stress response that may lead to psychiatric, immune, cardiovascular, and metabolic illnesses (McEwen, 1998).

Under normal conditions in humans a stimulus is processed as an acute stressor by the prefrontal cortex that initiates the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular division of the paraventricular nucleus (PVN) of the hypothalamus to induce the release of adrenocorticotropic hormone (ACTH) from the pituitary and into circulation where

ACTH then acts to elicit the release of cortisol from the adrenal glands (Sawchenko and Swanson, 1985; Swanson et al., 1983). As a glucocorticoid, cortisol mobilizes energy stores so that an individual can respond appropriately to a stressor. After the cessation of the perceived stressor, glucocorticoid negative feedback in limbic areas inhibits the release of cortisol and returns an individual to allostasis (McEwen, 2002). Data from rodents suggest that this negative feedback is due to glucocorticoids binding receptors at the level of the hippocampus, hypothalamus and pituitary to inhibit further release of CRH and ACTH, respectively (De Kloet and Reul, 1987; Keller-Wood and Dallman, 1984; Smith and Vale, 2006).

The prefrontal cortex (PFC) is critical in the regulation of the stress axis by initiating behavioral and physiological responses reflecting coping strategies for perceived stressors by mediating the activity of other limbic areas, including the hippocampus, amygdala, and hypothalamus (Compas, 2006; McEwen and Gianaros, 2010). Areas of the PFC, including the orbital and dorsal medial PFC, and the anterior cingulated cortex, communicate with the hypothalamus via indirect connections to initiate the release of corticotropin-releasing hormone (CRH) (McEwen and Gianaros, 2010). CRH is a 41 amino acid peptide that is primarily synthesized in the parvocellular subdivision of the PVN and acts as the primary central player in initiating the stress response (Rivier and Vale, 1983; Vale et al., 1981). CRH in rodents is expressed both in the central nervous system and in the periphery (Bale and Vale, 2004), with central expression of CRH localized primarily to the PVN, bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA) (Sawchenko and Swanson, 1985). CRH from the PVN is secreted into the portal vessels of the hypophysis that supply the

anterior lobe of the pituitary gland. By binding CRH Type 1 receptors (CRHR1) in pituitary corticotropes and activating the cyclic adenosine monophosphate (cAMP) pathway, CRH facilitates the release of ACTH into systemic circulation (Perrin and Vale, 1999; Rivier and Vale, 1983). ACTH stimulates the synthesis and release of glucocorticoids from the zona fasciculata of the adrenal cortex by binding melanocortin type 2 receptors (MC2Rs) (Mountjoy et al., 1992).

The primary glucocorticoid involved in the stress axis is cortisol in humans and in non-human primates and corticosterone in rodents. Glucocorticoids are steroid hormones that have an array of effects on physiology and behavior via binding of either mineralocorticoid receptors (MRs; Type 1) and/or glucocorticoid receptors (GRs; Type II). MRs and GRs are both intracellular steroid hormone receptors that homodimerize and translocate into the nucleus upon ligand binding where they bind DNA on glucocorticoid response elements (GREs) to affect gene transcription (Bamberger et al., 1996). Both GRs and MRs are involved in LHPA negative feedback (Coirini et al., 1985; Reul et al., 1990). Studies from rodents suggest that GRs in the hippocampus induce negative feedback of the LHPA axis following a stressor and restore homeostasis by inhibiting the PVN and downstream glucocorticoid release whereas MRs are involved in regulating diurnal glucocorticoid levels (De Kloet and Reul, 1987). Thus, the LHPA axis acts to respond to threats in a timely and acute manner such that homeostasis is restored following cessation of a perceived threat.

However, the constant hassles and struggles associated with everyday life can result in repetitive and prolonged activation of the LHPA axis and thus a chronic and prolonged exposure to stress hormones. Exposure to such chronic stress is typically

characterized physiologically by hypercortisolemia due to diminished glucocorticoid feedback. Chronic hypercortisolism is implicated in a number of psychopathologies, including depression (de Kloet et al., 2005; Holsboer, 2001; Keck et al., 2001). Dexamethasone is a widely used diagnostic tool to assess sensitivity to glucocorticoid negative feedback (Holsboer et al., 1994; Kalin et al., 1982). Decreased suppression of cortisol levels following dexamethasone administration indicate that glucocorticoids are less able to induce negative feedback and less effective in terminating LHPA axis responses. This reduced suppression of cortisol following the dexamethasone suppression test (DST) most often identifies individuals with major depressive disorder (Coryell et al., 2008; Jokinen et al., 2008) and indicates that the regulation of the LHPA axis within chronic stress environments is perturbed. Alternatively, individuals with post traumatic stress disorder exhibit LHPA dysregulation in the form of increased suppression of cortisol following a DST and decreased levels of basal cortisol (Meewisse et al., 2007; Yehuda et al., 2002).

Alterations in the expression of genes important for modulating the stress axis response to chronic stressors are implicated in the etiology of stress-induced disorders. Chronic stress exposure in rodents increases CRH expression in the CeA and the BNST (Albeck et al., 1997; Shepard et al., 2000) as well as the PVN (Ma et al., 1999). This is consistent with increased CRH expression in the PVN in people with depression (Raadsheer et al., 1994). Concurrent with the upregulation in CRH expression within the CeA is an increase in the expression of AVP in the PVN (Aguilera and Rabadan-Diehl, 2000; Ma et al., 1999; Makino et al., 1995) and downregulation or upregulation of CRHR1 in the PVN, depending on characteristics of the stressor (Bonaz and Rivest,

1998; Keen-Rhinehart et al., 2009). Allostasis is maintained by the decrease in CRH synthesis within the PVN that is critical for attenuating the stress response via glucocorticoid negative feedback (Aguilera et al., 2007). In contrast, the upregulation of CRH in the CeA under conditions of chronic stress results in the continued activation of the LHPA axis (Aguilera et al., 2007).

1.4 Genetics influence individual susceptibility to stress-induced disorders

Individual vulnerability to psychopathologies and other adverse stress-induced alterations in behavior and physiology can be influenced by genetics. The most well studied genetic locus that influences individual susceptibility to the adverse consequences of psychosocial stressor exposure is the polymorphism in the *SCL6A4* gene that encodes the serotonin reuptake transporter (5HTT) (Caspi et al., 2003). The short promoter length variant (s-variant) of 5HTT has reduced transcriptional activity in humans (Lesch et al., 1996) and individuals carrying this short promoter allele have a higher incidence of psychopathology (Caspi et al., 2003) than individuals homologous for the long 5HTT allele (l/l). Disruptions in the *SLC6A4* gene and 5HTT function are associated with a range of behavioral phenotypes, including increased anxiety, aggression, and impulsivity (Murphy and Lesch, 2008). Length variations in the 5HTT promoter are also present in rhesus monkeys (Bennett et al., 2002; Lesch et al., 1997) and are associated with reduced transcriptional activity. The s-variant polymorphism is also associated with maladaptive behavior in monkeys (Bennett et al., 2002) coincident with increased LHPA responsivity to stress (Murphy et al., 2001). Further, the long/short genotype produces a similar phenotype to that of the short/short genotype (Barr et al., 2004a; Barr et al., 2004b;

Champoux et al., 2002). Importantly, the s-variant of the 5HTT gene interacts with the environment throughout one's lifespan to affect physiology and behavior. Studies in monkeys have shown that peer- but not mother-reared infant rhesus monkeys with the s-allele have lower CSF levels of 5HIAA, the 5HT metabolite, (Bennett et al., 2002), show a greater ACTH response to social separation (Barr et al., 2004b), engage in less play, and show more emotional distress (Barr et al., 2004b; Bethea et al., 2004; Champoux et al., 2002).

Genetic factors other than the 5HTT polymorphism within the serotonergic system have been associated with increased individual vulnerability to stress-induced disorders. A single nucleotide polymorphism (SNP) within the gene encoding the 5HT autoreceptor 5HT1A (*Htr1a*), is linked to poor affect, including depression, anxiety, and borderline personality disorder (Albert and Lemonde, 2004; Cowen et al., 1994; Hansenne et al., 2002; Lesch and Gutknecht, 2004; Savitz et al., 2009), as well as personality traits such as neuroticism (Strobel et al., 2003). Both normal and depressed individuals carrying the 5HT1A SNP show region-specific alterations in 5HT1A binding (Drevets et al., 1999; Parsey et al., 2006). Importantly, the 5HT1A SNP is also associated with decreased response to antidepressants (Lemonde et al., 2004; Lesch et al., 1990; Lesch et al., 1991), altered reactivity of the amygdala towards threat-related stimuli and increased trait anxiety (Fakra et al., 2009; Le Francois et al., 2008). Genetic variation in 5HT receptors genes is not limited to 5HT1A, but also has been described in the gene encoding the 5HT2A receptors. A SNP in the gene encoding for 5HT2A receptor is implicated in the vulnerability to depression, posttraumatic stress disorder, and panic disorder (Choi et al., 2004; Inada et al., 2003; Lee, 2007). Furthermore, SNPs

within the gene encoding for tryptophan hydroxylase (TPH2), the enzyme critical for the synthesis of serotonin within the brain, have been associated with affective disorders (Harvey et al., 2004; Zill et al., 2004a; Zill et al., 2004b), suicidality (Jollant et al., 2007; Ke et al., 2006; Lopez et al., 2007) and self-harm (Pooley et al., 2003), as well as borderline personality disorder (Wilson et al., 2009), panic disorder (Maron et al., 2007), attention deficit hyperactivity disorder (Gutknecht et al., 2007; Walitza et al., 2005), and behavioral traits related to emotional instability (Gutknecht et al., 2007). Regulation of TPH is mediated via an array of hormones and stressors, including glucocorticoids upon activation the LHPA axis (Malek et al., 2007), suggesting an environmental influence upon expression of TPH. Indeed, adverse early life experience modulates TPH2 expression (Gardner et al., 2009; Mueller and Bale, 2008), and the presence of the TPH2 SNP in monkeys exacerbates the negative effects of adverse rearing on both serotonergic and LHPA function (Chen et al., 2010).

Other polymorphisms that extend beyond the serotonergic system are associated with individual susceptibility to stress-induced disorders. A variable number of tandem-repeat (VNTR) polymorphism in the monoamine oxidase A (MAOA) gene, an enzyme critical for the degradation of serotonin and other monoamines, influences expression levels of MAOA, as some gene variants are ten times more efficient in gene transcription than others (Sabol et al., 1998). The low variant VNTRs in the MAOA gene are associated with increased incidence of depression, poor sleep quality (Brummett et al., 2007; Gutierrez et al., 2004) and an altered response to stressors (Jabbi et al., 2007). The MAOA genetic locus interacts with the environment to increase vulnerability to behavioral disruptions, as adversely reared monkeys carrying one of the low MAOA

variants show increased aggression and anxiety-like behavior (Karere et al., 2009; Newman et al., 2005). Additionally, the effects on these low expression variants of the MAOA gene interact with a SNP in the gene encoding for COMT, an enzyme primarily involved with deactivation of dopamine (Yavich et al., 2007), to yield increased ACTH and cortisol in response to a Dex/CRH challenge as well as increased suicide attempts (De Luca et al., 2006; Jabbi et al., 2007) and increased incidence of depression during the peripartum period (Doornbos et al., 2009).

Variation within genes whose expression are critical for the functioning of the LHPA axis have also been implicated in increasing individual vulnerability to stress-induced disorders. Polymorphisms in the glucocorticoid receptor (GR) are associated with both increases and decreases in glucocorticoid sensitivity, both of which are linked to the etiology of depression (Claes, 2009; van Rossum et al., 2002). The ER22/23EK SNP in the GR gene interacts adversely with childhood abuse to increase the probability that one will suffer from recurrent depressive symptoms (Bet et al., 2009). Individuals with this same GR SNP also show a faster clinical response to antidepressants (van Rossum et al., 2006), suggesting that genetics can influence the efficacy of pharmacological interventions. Hypercortisolemia in stress-induced disorders is in some cases due to increased activity of CRH neurons within LHPA circuitry (Nemeroff et al., 1984; Reul and Holsboer, 2002), suggesting that genetic factors regulating CRH and its receptors might influence individual vulnerability to stressors. Indeed, a functional SNP in the gene encoding for CRH interacts with adverse early experience in monkeys to heighten stress-induced LHPA activity and alcohol consumption, and decrease environmental exploration (Barr et al., 2009). Polymorphisms in the gene encoding CRH

type 1 receptor, CRHR1, have also been associated with stress-induced disorders, including major depression (Licinio et al., 2004; Liu et al., 2006). Individuals carrying specific CRHR1 SNPs who were abused as children are more likely to develop depressive symptoms and increased cortisol response to a Dex/CRH challenge than individuals carrying these same alleles who did not experience childhood abuse (Bradley et al., 2008; Polanczyk et al., 2009; Tyrka et al., 2009). Interestingly, a sex difference is associated with the CRHR1 gene by environment interaction, as only women carrying the rs110402 allele of the CRHR1 gene who were abused as children show increased incidence of depression later on in adulthood (Heim et al., 2009a; Heim et al., 2009b). These data provide specific genetic evidence for why depression is more heritable in women than men (Kendler et al., 2006) and might thus influence the mechanisms by which women are twice as susceptible as men to stress-induced disorders.

While we have discussed only a subset of genetic loci that play crucial roles in the regulation of the LHPA axis and thus influence individual response to stressor exposure, it is beyond the scope of this dissertation to summarize them all. We have highlighted polymorphisms that are associated with stress-induced affective disorders because they might also be associated with adverse consequences associated with stress-induced phenotypes in females. Even though one-gene associations are most commonly described, we must not forget that differences in individual stress responsivity is likely polygenic. The complex nature of these genetic influences reminds us that it is extremely difficult to truly discern the effects of environment on one-gene causality of such complex disorders. Regardless of this, there is a clear association between adverse environments and genetics that influence individual vulnerability to unfavorable health

consequences. While the mechanism by which adverse experience during early life modifies the genome is dependent upon epigenetic alterations to the chromatic structure of DNA and not the DNA sequence itself (Weaver, 2007), it is difficult to study such gene by environment interactions in animal models currently employed to study how chronic psychosocial stressor exposure affects behavior and physiology.

1.5 Animal models of chronic exposure to stressors

Animal models are crucial for understanding not only neurochemical changes caused by chronic stress exposure discussed above, but also for delineating the adverse health consequences that result under such conditions. The suitability of an animal model as a representation of conditions and experiences typical of humans is determined by whether the animal model has face, construct and predictive validity (Albelda and Joel, 2011; Modi and Young, 2011). Face validity addresses whether the behavior or physiology studied with an animal parallels that seen in humans. Construct validity is achieved when there is a similar underlying etiology of behavioral and physiological processes in both humans and the animal model being employed. Predictive validity addresses whether pharmacological and behavioral approaches used in an animal model predict what is seen or might be seen in human populations. Thus, animal models of chronic stress exposure would have face, construct, and predictive validity if the models 1) employed a type of stressor that is similar to the types of stressors experienced by humans and have similar consequences as those seen in humans, 2) affect behavior and physiology in humans and the animal species in similar etiological manners, and 3) produce similar pharmacological and behavioral responses to interventions as seen in

human populations. An animal model that attains concurrent face, construct, and predictive validity will provide a translational approach with which to study the etiology of conditions experienced by humans in a relevant ethological manner.

One approach undertaken to emulate the consequences of chronic stress without subjecting animals to actual physical or emotional stressors has been exogenous administration of high levels of glucocorticoids to mimic elevated levels that are associated with chronic exposure to stressors in rodents (Akana et al., 1996; Bhatnagar and Meaney, 1995; Ottenweller et al., 1989) and individuals diagnosed with depression (Holsboer, 2001). Chronic corticosterone administration in male rats over three weeks increases adrenal size and alters endogenous ACTH and corticosterone (Donner et al., 2011). Furthermore, chronic corticosterone treatment increases both anxiety- and depressive-like behavior in a dose dependent manner as assessed by open field, elevated plus and forced swim tests (Donner et al., 2011). Dysregulation of the serotonergic system similar to those seen in humans with depression are also induced by exogenous corticosterone administration (Donner et al., 2011).

A similar approach to induce a condition that resembles chronic stress exposure by a physiological intervention has been achieved by overexpressing CRH site-specifically in the CeA of female rats using a lentivirus vector (Keen-Rhinehart et al., 2009; Regev et al., 2011). Overexpression of CRH in the CeA not only mimics the increases expression of CRH in CeA in rodents associated with chronic stress exposure (Albeck et al., 1997; Shepard et al., 2000), but also has adverse effects on LHPA and reproductive physiology, as well as behavior (Keen-Rhinehart et al., 2009). Specifically, CRH-injected females show diminished glucocorticoid negative feedback as well as

compromises in reproductive physiology as evidenced by a lengthening of the ovarian cycle and decreased expression of GnRH in the medial preoptic area of the hypothalamus. Motivational and emotional behaviors are also altered by CRH overexpression in the CeA, as motivation to engage in sexual behavior is diminished, anxiety-like behaviors are augmented in an acoustic startle task and depressive-like behavior increased as assessed by the forced swim test (Keen-Rhinehart et al., 2009).

While both the above-mentioned models have predictive validity concerning the performance of the treated animals on behavioral paradigms that have predictive validity in screening for antidepressants and anxiolytics (Castagne et al., 2011), these models are lacking in face validity because an actual stressor was not used to induce the adverse consequences associated with chronic stress exposure. Rather the strength of these models stems from their construct validity, as both increased expression of CRH in the CeA and increased levels of peripheral glucocorticoids parallel what is seen following chronic stress exposure (Akana et al., 1996; Albeck et al., 1997) and leads to behavioral and other physiological changes that parallel adverse consequences of chronic stress exposure in humans, including altered metabolic state (Simon and Arterburn, 2009; Werrij et al., 2006), reproductive compromise (Wainwright et al., 2011) and increased behavioral disruption (Oroszi and Goldman, 2004; Sinha, 2008). However, by omitting the use of an exposure to stressors, these models cannot be used to study the mechanism responsible for the overexpression of CRH in the CeA and increase in glucocorticoids that are important in the etiology of stress-induced diseases.

Many other rodent models of chronic stress exposure employ the use of physical and psychosocial stressors to induce physiological and behavioral states similar to

characteristics of stress-induced disorders in humans. Immobilization or restraint stress is a common model for chronic stress exposure in rodents. Animals are placed in plastic tubes that immobilize their lateral and forward movement, typically for 30 minutes a day, for 7 consecutive days (Akana et al., 1992; Strausbaugh et al., 1999). Daily restraint for 7 consecutive days increases corticosterone levels after subsequent bouts of restraint as well as ACTH levels (Dallman et al., 2004a). Animals that undergo chronic restraint also show alterations in metabolic phenotype, including a decrease in body weight, an effect that is typically perceived as a confirmation that restraint is indeed stressful to rodents (Strausbaugh et al., 1999). Studies using restraint in rats have shown that the immune system is compromised in restrained animals, similar to what is observed in humans when daily stressors exacerbate the symptoms of immune disorders, such as rheumatoid arthritis (Zautra et al., 1997) and asthma (Sekas and Wile, 1980). More recently, the chronic restraint model in rodents has been critical studying how dietary environment and access to diets high in fat and sugars interacts with stress exposure to alter feeding behavior and metabolism (Foster et al., 2009; Pecoraro et al., 2004), similar to consequences of chronic stressor exposure observed in humans (Dallman et al., 2004b).

Another model of physical chronic stress exposure in rodents is chronic intermittent cold stress exposure where animals are subjected to a few hours of 4-6°C a day, for a week. The neurochemical and neuroanatomical methods used concurrently with this model have been critical in elucidating the underlying circuits that are activated by acute stress superimposed on a background of chronic cold stress, namely the posterior paraventricular hypothalamus to the thalamus-amygdala to the parvocellular PVN circuit (Bhatnagar and Dallman, 1998; Bhatnagar et al., 2000). Additionally,

Dallman and colleagues have employed chronic cold stress exposure to determine how glucocorticoids interact with a chronic stress background to modulate the activity of the LHPA axis in response to subsequent stressors (Akana and Dallman, 1997). Additionally, combining cold stress with corticosterone administration in a site-specific manner has elucidated differential roles of the prefrontal cortex and CeA in modulating LHPA activity (Akana et al., 2001).

Even though physiological and physical stress paradigms have been developed to examine how chronic stress may produce negative health outcomes, for the most part these models elicit behavioral and hormonal responses that are unique to the particular type of stress employed in the laboratory setting. Although these studies are important from a heuristic point of view and are certainly informative, the animal models they employ lack face and construct validity. Any investigation of the biobehavioral effects of stress as it relates to the development of human pathophysiology should focus on those stressors that are likely to be shared by human populations (Anisman and Matheson, 2005; Foster et al., 2006; Tamashiro et al., 2005). Moreover, in many models of chronic stressor exposure animals, including restraint stress and corticosterone administration, eventually adapt to stressors and do not continue to exhibit stress hormone or behavioral responses (Armario, 2006; Bhatnagar and Dallman, 1998; Bhatnagar et al., 1998; Bhatnagar and Vining, 2003; Bhatnagar et al., 2006; Jaferi and Bhatnagar, 2006). However, when the chronic stress is uncontrollable, and/or not easily predictable then both the sympathetic and neuroendocrine responses are continually re-activated and sustained, inducing physiological and behavioral changes.

Several rodent models of chronic stress induce sustained neurobiological and behavioral changes that resemble stress-induced disorders in people via psychosocial stressors that mimic human experiences. The chronic variable stress (CVS) paradigm (Herman et al., 1995) exposes rats to 6 weeks of repeated mild stressors, including physical stressors (restraint, home cage tilt, cold stress) and psychosocial stressors (social isolation). This CVS paradigm produces animals that show increased basal corticosterone and ACTH, as well as increased adrenal size. Both CRH and AVP levels within the PVN in animals subjected to this CVS are increased (Herman et al., 1995). These changes in signal expression occur in parallel with a decrease in MR and GR expression in the hippocampus as well as the PVN of these animals (Herman et al., 1995). The dysfunction within the LHPA axis in this model is associated with altered behavioral phenotypes as well, similar to those seen in humans suffering from psychopathologies such as depression. Fear learning and emotional arousal are enhanced altered in animals subjected to CVS (McGuire et al., 2010). Additionally, rats subjected to CVS show anhedonia (Dalla et al., 2005; Flak et al., 2009) and altered appetite, metabolism, and activity following presentation of a diet high in fats and sugar (Solomon et al., 2011). The CVS model has been very useful in elucidating how specific limbic structures, such as the BNST (Choi et al., 2007), and neurochemical signals (McGuire et al., 2011; Ostrander et al., 2009), alter their function in response to acute and chronic stressors by using neurochemical and neuroanatomical techniques. Furthermore, this rodent model of unpredictable chronic stress exposure is one of the few models that have been used to study sex differences in the development of the responses to CVS and the

underlying mechanisms that might be responsible for these differences (Carvalho-Netto et al., 2011; Solomon et al., 2011).

Two other rodent models employ the use of an ethological social stressor as the central component. In the social defeat model, most typically studied in hamsters, repeated exposure to a more aggressive intruder on a single day results in the subordination of the socially defeated animal (Raab, 1986). Social defeat produces sustained activation of the LHPA axis (Raab, 1986; Razzoli et al., 2009), immune dysfunction (Stefanski, 2000) and specific changes in neurochemical circuits within mesolimbic regions in both male and female rodents (Foster et al., 2006; Razzoli et al., 2009). Behaviorally, social defeat is associated with anhedonia, decreased activity and sociality, and increased depressive-like behaviors as assessed by the forced swim test (Razzoli et al., 2009). Additionally, reproductive function is inhibited (Sugiura et al., 1997) and food intake and body weight are attenuated (Meerlo et al., 1997). The submissive behavior persists ~50% in males for up to a month (Huhman et al., 2003) whereas response to defeat is diminished in females. Indeed, more submissive behavior is expressed during estrous cycle days associated with elevated estradiol concentrations (Solomon et al., 2007).

Finally, in the visible burrow system (VBS), groups of male rats are housed socially with several females for two weeks. Males quickly form a dominance hierarchy and subordinate males exhibit a number of changes characteristic of chronic stress exposure. Neurobiological changes in limbic circuits are evident in subordinate males, including decreases in expression of MRs and GRs within the hippocampus (Chao et al., 1993) and increases CRH within the BNST (Choi et al., 2006) and the CeA (Albeck et

al., 1997). Basal corticosterone levels and adrenal size are increased (Blanchard et al., 1995) in subordinates that also show reproductive deficits (Hardy et al., 2002; Monder et al., 1994) concurrently with alterations in the dopaminergic system (Lucas et al., 2004). Subordinate males have attenuated body weight that is attributable to decrease adipose tissue that results in hypoleptinemia (Blanchard et al., 1993; Blanchard et al., 1995; Choi et al., 2006; Tamashiro et al., 2004).

Additionally, the VBS model has several notable features. First, animals are given intermittent recovery periods from social housing during which previously subordinate males respond differently than dominant males, most typically with excess food intake and weight gain (Tamashiro et al., 2004). However, the subordinate phenotype is maintained with re-exposure to the social housing (Lucas et al., 2004; Tamashiro et al., 2007). Secondly, a subgroup of subordinate males is classified as non-responders, showing a diminished response in corticosterone to an acute restraint during the social housing period (Lucas et al., 2004; Watanabe et al., 1995), a profile analogous to the attenuated stress hormone activity described for post traumatic stress disorder (Meewisse et al., 2007; Yehuda et al., 2002). Importantly, despite this reduced glucocorticoid response, these males show more altered dopaminergic tone in mesolimbic regions than other subordinate or dominate animals (Lucas et al., 2004). However, because females of the strain of rats used do not form a hierarchy when housed socially, this paradigm cannot be used to evaluate adverse consequences of chronic social stress in females (Tamashiro et al., 2004).

Together, the CVS, social defeat, and VBS models of chronic stress exposure employ psychosocial methods that give these animal models both face and construct

validity because on the surface the stressors employ similar stressors to those experienced by humans that have similar physiological effects that are important in the development of adverse chronic-stress states. Importantly, these animal models represent well-established approaches that have significantly advanced our understanding of how chronic exposure to social stressors produces lasting changes in behavior and physiology. However, while each of these paradigms employs a repeated uncontrollable or unpredictable type of stressor, some face and construct validity is lost because the stressor is discontinued after a specific duration of time. Thus, these paradigms only partially model the continual daily exposure to stressors experienced by people and implicated in the development of stress-induced disorders.

Another important consideration for the study of stress-induced alterations in physiology and behavior that is often neglected in animal models is the sex of the subjects. This is an important consideration when in humans women are twice as likely as men to suffer from stress-induced disorders, including depression, anxiety, emotional feeding and obesity (Barry et al., 2008; Jones and Carney, 2006; Weissman and Olfson, 1995; Wurtman, 1993; Wurtman and Wurtman, 1995; Zellner et al., 2006; Zellner et al., 2007). Both sex chromosomes and gonadal steroids influence sex difference in behavior and physiology (Goldstein et al., 2010). From the models discussed previously, only two of the models (CRH overexpression in the CeA and social defeat) were used to assess stress-induced alterations in behavior and physiology in females. The lack animal models that address the ever present sex difference in the etiology of stress-induced disorders necessitates the development of an ethologically relevant model of chronic unpredictable exposure specifically in females.

1.6 Social subordination in female rhesus monkeys

Socially housed macaque monkeys provide an opportunity to study the impact of continual exposure to social stress over an extended period of time on a number of health-related phenotypes in females (Sapolsky, 2005). Matrilineal dominance hierarchies organize macaque social groups, regardless of group size, and function to maintain group stability. Dominance is asserted by contact aggression and non-contact aggression that results in harassment. While an animal's position within the hierarchy can be enforced through contact aggression, most often subordinate status is imposed by the threat of aggression or harassment from more dominant animals (Bernstein, 1976; Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984). Subordinate females terminate these agonistic interactions by emitting submissive behavior, which is the defining feature of social subordination in macaque groups (Bernstein, 1976; Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984). The nature of aggression is most often random and unprovoked, providing uncertainty to when these attacks might occur, thus increasing the cost of this harassment on subordinate individuals (Silk, 2002). This frequency and unpredictability of harassment reduces the control over an individual's social and physical environment in more subordinate animals (Bernstein and Mason, 1970).

Several unique features differentiate this social subordination model of chronic stressor exposure from more typical laboratory animal paradigms. Because the social housing mimics the organization of free-ranging populations, the stress of subordination is a part of this species' natural history, thus providing ethological validity to studying the consequences of subordination on health in these animals (Bernstein and Gordon, 1977).

Secondly, matrilineal relations define the dominance hierarchy and is thus female-based (Bernstein and Mason, 1970), providing an important opportunity to study stress-induced alterations in behavior and physiology specifically in females. Furthermore, social groups are stable for extended periods, even when experimentally established (Jarrell et al., 2008), providing the opportunity to study the long-term consequences of either high or low social status on behavior and physiology (Kaplan, 2008).

The social subordination model in macaques is being used to study the adverse effects of psychosocial stress on a range of health related conditions known to be stress dependent (Troisi, 2002), including cardiovascular disease (Kaplan et al., 2009), addictive behavior (Morgan et al., 2002), reproductive compromise (Kaplan and Manuck, 2004; Wilson et al., 2008; Zehr et al., 2005), immune dysfunction (Gust et al., 1991; Paiardini et al., 2009), and an increase in emotionality (Shively et al., 2005; Wilson et al., 2008). However, even though the recurrent exposure to harassment from more dominant females leads to larger adrenal glands (Kaplan et al., 1984), greater cortisol response to social challenges (Cohen, 1999), diminished glucocorticoid negative feedback (Jarrell et al., 2008; Shively, 1998b; Wilson et al., 2008) and altered responses to ACTH (Shively, 1998b) in subordinate females, a full characterization of how these alterations in stress physiology affect socio-emotional behavior, the reproductive axis and metabolic health remains to be determined.

1.7 Overall goal and rationale

The focus of this dissertation is to delineate the changes that occur in LHPA regulation due to social subordination and determine whether these differences produce

distinct behavioral and physiological phenotypes that are known to be stress dependent in females. A secondary emphasis was to assess how the 5HTT polymorphism influences individual vulnerability to adverse consequences of psychosocial stressor exposure. To address our overall aim, we evaluated the effects of social subordination and 5HTT genotype on four modalities, including stress axis function (Chapter 2), social and anxiety-like behavior in the presence of the ovarian hormone estradiol (Chapter 3), food intake and metabolic profile under specific dietary conditions (Chapter 4), and reproductive physiology in the presence of estradiol (Chapter 5). We hypothesize that social subordination in female rhesus monkeys dysregulates LHPA activity, decreases sensitivity to the socioemotional effects of estradiol, alters feeding behavior in complex dietary environments, and disrupts reproductive function. Furthermore, we hypothesize that the presence of the s-variant allele of the 5HTT gene exacerbates the consequences of social subordination in females. Finally, in Chapter 6, we perform a discriminate analysis on variables collected throughout this dissertation work that provides evidence that social subordination results in two distinct phenotypes that we can study to assess how psychosocial stress exposure affects behavior and physiology in females.

1.8 General material and methods

Subjects. All subjects were ovariectomized adult female rhesus monkeys (*Macaca mulatta*) aged between 9 – 13 years. Subjects were housed in one of eight small social groups at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA). Groups were comprised of 5 females and one adult male (unless otherwise specified) and were maintained in run-type enclosures having an indoor and

outdoor area that measured 20 x 15 x 8 feet each. Animals were fed a commercial low fat, high fiber monkey diet (Purina Mills, St. Louis Mo) *ad libitum* twice daily, supplemented daily by seasonal fruit and vegetables, unless otherwise specified. All procedures described in this dissertation were approved by the Emory University Animal Care and Use Committee in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for Care and Use of Laboratory Animals."

Ovariectomy. Bilateral ovariectomy occurred in all females 12 months prior to initiation of the described dissertation studies by laparoscopy. Following anesthesia with ketamine and isoflurane, a 1 cm incision was made through the skin, several centimeters proximal to the umbilicus. Two, small cannulas were inserted, one on either side of the lower abdomen for tissue manipulation with instruments and each ovary visualized and isolated with forceps. Vascular clips were then introduced through the other cannula and applied around the ovary. The ovary was cut free and removed through the opening with the forceps and cannula. The same procedure was performed on each side. Females averaged approximately 2.73 ± 0.31 pregnancies prior to ovariectomy procedure.

SCL6A4 Genotyping. A blood sample was collected and DNA extracted from blood using the Pure Gene Blood Kit (Gentra, D-4000). Gene amplification via polymerase chain reaction (PCR) identified polymorphisms in the 5HTT promoter region (5HTTLPR) using the forward and reverse primers, "*cag ggg aga tcc tgg gag gga"* and "*ggc gtt gcc gct ctg aat gc"* respectively. The short allele amplicon was identified at 398 base pairs and the long allele amplicon at a length of 419 base pairs by direct visualization using an ethidium bromide agarose gel. A total of 179 females were

screened for 5HTT allele status in nine breeding groups at the YNPRC. Of the 179 females, 50.2% had the l/l genotype, 45.3% had the heterozygous l/s genotype and 4.50% had the homozygous s/s genotype (Hoffman et al., 2007).

Group formation. The eight groups of animals used in these studies were formed by removing subjects from their natal groups and randomly introducing unfamiliar animals consecutively to each other to form new groups. Two unfamiliar animals were placed together in two small adjacent indoor-outdoor enclosures and allowed to acclimate to each other for 24 hours through a mesh screen that allowed only for visual and olfactory sensation. Following these 24 hours, the two females were placed in the same run and allowed to established dominance. A third female was then placed into the adjacent run where she had only visual access to the previously established pair of females. Following 24 hours, the third female was introduced to the initial pair. This procedure was repeated until five females were living together in a single in indoor-outdoor run (Jarrell et al., 2008). Three years after the formation of these all-female groups, introductions of adult males were undertaken. Each male was placed in an adjacent indoor-outdoor enclosure to that of a group of females and allowed to acclimate to each other for 24 hours through a mesh screen that allowed only for visual and olfactory sensation. Following these 24 hours, the male was introduced to the females.

Females were selected from their natal groups based on parity, 5HTTLPR genotype and dominance status within natal groups. At the time of selection, all females were mulitparous and aged between seven and ten years. Females selected came from the middle portion of the social dominance hierarchy within their natal groups to control for any previous exposure to stressors subjects might have encountered due to their social

status in these natal groups. Experimental groups were formed homogenous for genotype, to ensure that each social status position had representation of both 5HTT genotypes. Thus, four groups of females were all of the l/l 5HTTLPR genotype and four groups of females of the s-variant 5HTTLPR genotype.

Classification of social status. The imposition of social subordination is accomplished through contact aggression and continual harassment and the threat of aggression (Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984). Thus, social status is determined empirically based on the outcome of dyadic interactions in which a female clearly emits a submissive response to another animal (Bernstein, 1976). A female who clearly emitted an unequivocal submissive behavior in response to another female was ranked lower than the female to whom she was submitting. In chapter 2 we validate using previously established conventions to categorize females as either dominant or subordinate (Kaplan et al., 1984). In all the studies presented in this document, females ranked 1or 2 in their group were classified as dominant and those ranked 3 or below were considered subordinate.

Behavioral observations. Data were recorded using a Palm PDA or an Acer Netwbook and the "Hands Obs" or "Win Obs" program developed by the Center for Behavioral Neuroscience (Graves and Wallen, 2006). Data were collected in the format of actor – behavior – recipient. Inter-observer reliability exceeded 90%. Group behavioral observations were performed with a single observer collecting data on all animals in a social group at the same time. Each behavioral observation collected was 30 minutes in duration. An established behavioral ethogram was used to document agonistic, affiliative, and anxiety-like behaviors (Jarrell et al., 2008). Affiliative behavior

comprised of proximity and grooming; aggression was defined by contact and non-contact threats, slaps, grabs, and bites; and submissive behavior was characterized by withdrawals, grimaces, and screams. Anxiety-like behavior consisted of body shakes, yawns, and self-scratching (Troisi, 2002).

Sampling. All subjects were habituated to being removed from their group for venipuncture. Samples were generally obtained within 10 to 15 minutes from entering the animal area to minimize arousal. This approach reliably results in lower baseline cortisol measures in capture-acclimated monkeys (Blank et al., 1983) and does not compromise parameters of reproduction (Walker et al., 1982). Samples were centrifuged at 3000 RPM and serum immediately stored at -20 degrees C in cryovials until time of assay.

Hormone assays. Serum levels of estradiol were assayed using a modification of a previously validated commercial assay (Siemens/DPC; Los Angeles CA) (Pazol et al., 2004). Using 200 µl of serum, the assay has a sensitivity of 5 pg/ml and an intra- and inter-assay coefficient of variation (CV) of 5.2% and 11.1%, respectively. Serum levels of cortisol were determined by radioimmunoassay (RIA) with a commercially available kit (Beckman-Coulter/DSL, Webster TX) previously described for rhesus monkeys (Stavisky et al., 2001; Wilson et al., 2005; Winslow et al., 2003). Using 25 µl, the assay has a range from 0.5 to 60 µg/dl with an inter- and intra-assay CV of 4.9% and 8.7%, respectively. Serum concentrations of luteinizing hormone (LH) were measured by radioimmunoassay using reagents provide by the NIDDK-National Hormone & Peptide Program (Harbor-UCLA Medical Center, Torrance CA) that has been previously validated (El Majdoubi et al., 2000). The assay uses macaque LH as the standard and

antiserum directed against macaque LH. Using 100 µl of serum the assay has a range from 0.2 to 10 ng/ml and an inter- and intra-assay CV of 4.9% and 8.7%, respectively. Immunoreactive serum oxytocin was measured by EIA using a kit distributed by Enzo Life Sciences from Assay Designs (Ann Arbor MI). This assay measures immunoreactive oxytocin across a number of species in peripheral samples or cerebrospinal fluid. For rhesus monkeys, 50 µl of serum is diluted in 150 µl of assay buffer. With this protocol, the assay has a sensitivity of 15.6 pg/ml with an inter- and intra-assay CV of 7.5% and 10.2%, respectively.

Serum leptin was measured by a RIA using a commercially available kit (Millipore, St. Louis MO). Assaying 100 µl, the assay has a range of 0.5 to 100 ng/ml. Intra-assay CVs were 6.84% and inter-assay were 7.24% (Wilson et al., 2003). Serum insulin was assayed with a RIA kit from Siemens having a sensitivity of 3 to 372 IU/L and an inter-and intra-assay CV of 9.02%and 5.87%, respectively (Jarrell et al., 2008). Serum glucose was determined by a commercially available colorimetric enzyme assay (Stanbio Laboratory, Boerne TX), having a range from 0 to 27 mmol/L and inter-and intra-assay CVs of 2.12% and 4.21%, respectively (Jarrell et al., 2008).

Dexamethasone quantification was achieved using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) on a Thermo Scientific LTQ-Orbitrap mass spectrometer and Surveyor HPLC system. Calibrators of dexamethasone-spiked rhesus macaque serum were employed to define the standard curve and assay range. All samples and calibrators were spiked with 20 ng of flumethasone and subjected to diethyl ether liquid-liquid extraction. Extracts were subsequently evaporated to dryness under nitrogen at 37 ºC and resolubilized for LC-MS/MS analysis in 200 µL of

30:70 water/methanol. The LC-MS/MS analysis was performed by negative ion APCI with SRM (selected reaction monitoring) detection and reverse phase chromatography. Chromatographic separations were accomplished using a linear gradient from 40:60 5 mM ammonium acetate/methanol to 100% methanol on a Supelco Discovery C8 column (50 x 2.1 mm, 5 μm particles). Using 200 μl, the assay has a range from 1 to 50 ng/ml with an inter- and intra-assay CV of 3.32% and 4.09%

Except for the fructosamine and PACAP analyses, all assays were performed in the Yerkes Biomarkers Core Lab. Antech Diagnostics (Atlanta GA) performed the fructosamine assay. Dr. Victor May (University of Vermont) performed the assay of serum PACAP.

CHAPTER TWO:

SOCIAL SUBORDINATION IMPAIRS HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION IN FEMALE RHESUS MONKEYS

2.1 Abstract

Linear dominance hierarchies organize and maintain stability in female rhesus macaque social groups regardless of group size. As a consequence of their low social status, subordinate females suffer from an array of adverse outcomes. However, data that differentiate limbic-hypothalamic-pituitary adrenal axis (LHPA) parameters between dominant from subordinate female monkeys are inconsistent, bringing into question whether social subordination dysregulates the LHPA axis in female macaques and thus whether it is truly a valid model of psychosocial stress exposure. One difficulty in examining LHPA function in macaques may be the confounding effects of cycling ovarian steroids that are known to modulate LHPA activity. The current study used ovariectomized dominant and subordinate female rhesus monkeys to examine the effect that social subordination has on LHPA function by measuring morning serum cortisol levels, dexamethasone (Dex) suppression of cortisol, metabolic clearance of Dex, and ACTH stimulation of adrenal cortisol release. Compared to dominant females, subordinate females showed blunted morning cortisol secretion, diminished glucocorticoid negative feedback, and decreased adrenal cortisol response to an ACTH challenge. However, the metabolism of Dex did not account for differences in Dex suppression between dominant and subordinate females. These results indicate that the ability to mount and limit glucocorticoid release is significantly reduced by psychosocial stress in female rhesus macaques, suggesting a hyporesponsive LHPA phenotype which resembles that observed in several human psychopathologies.

2.2 Introduction

The psychogenic component of chronic stress is implicated in the development of a number of adverse health outcomes in human beings including depression and anxiety illnesses, drug addiction and obesity (Juster et al., 2010; McEwen, 2008; Oroszi and Goldman, 2004; Pasquali and Vicennati, 2000; Sinha, 2008). Exposure to psychogenic stressors involves activation of cortico-limbic circuits that modulate both sympathetic and limbic-hypothalamic pituitary-adrenal (LHPA) responses (Choi et al., 2008; Herman et al., 2003; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). Whereas acute physical or psychological stress initiates a coordinated sequence of responses to manage energy resources and ultimately restore homeostasis (McEwen; Schulkin et al., 1994), the chronic unpredictable nature of certain stressors can overwhelm these reactive and restorative mechanisms and result in dysregulation of central and peripheral circuits regulating stress and behavioral responses (McEwen, 1998).

Many paradigms involving laboratory animals have been developed to examine how chronic stress may produce negative health outcomes. However, many of these animal models lack a psychosocial component and/or consist of repetitive and predicable procedures in which animals eventually adapt and do not continue to exhibit stress hormone or behavioral responses to that stressor (Armario, 2006; Bhatnagar and Dallman, 1998; Bhatnagar and Vining, 2003; Bhatnagar et al., 2004; Bhatnagar et al., 2006; Jaferi and Bhatnagar, 2006). Therefore, while interesting from a functional perspective, these paradigms do not reproduce the effects of chronic stress as it is most often experienced by human beings, that being psychosocial and uncontrollable or unpredictable in nature (Tamashiro et al., 2005). For instance, the experiences of people

in war and in low socio-economic conditions are two examples that typify the human experience of chronic stress. Certainly, these two examples of chronic stress that affect people are related to a range of psychopathologies (reviewed in (Breslau, 2001; Gallo et al., 2004; Isovaara et al., 2006; Lemaire et al., 1994; Lemstra et al., 2009; McEwen, 2000). Hence, animal models developed to approximate chronic stress as experienced by humans, and intended to study the etiology of human psychopathologies, should have a strong psychosocial component (Anisman and Matheson, 2005; Huhman, 2006), and produce the type of stress similar to that encountered by people (Tamashiro et al., 2005).

We propose that socially housed macaque monkeys provide an ethologically relevant opportunity to study the impact of chronic psychosocial stress on a number of health-related outcomes (Sapolsky, 2005). When housed socially, female macaques organize themselves into a linear dominance hierarchy wherein subordinate members are under constant harassment by more dominant animals (Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984) and have less control over their social and physical environments (Bernstein, 1976; Sapolsky, 2005). Subordinate animals suffer from an array of maladies that are though to be stress dependent, including reproductive compromise (Kaplan et al., 2010), altered pubertal timing (Zehr et al; Wilson et al 1986; Wilson & Kinkead 2008), emotional feeding (Arce et al., 2010); immune dysfunction (Paiardini et al., 2009); altered reward pathways and psychostimulant self-administration (Grant et al., 1998; Morgan et al., 2002); and cardiovascular health (Kaplan and Manuck, 1999). Despite this, questions remain as to the specific stress hormone phenotype exhibited by subordinate compared to dominant female rhesus monkeys. This issue is important because a number of LHPA parameters do not consistently differentiate

dominant versus subordinate females, notably morning cortisol (Czoty et al., 2009; Gust et al., 1993; Stavisky et al., 2001), diurnal cortisol (Arce et al., 2010; Collura et al., 2009), or the response to adrenocorticotropic hormone (ACTH) (Riddick et al., 2009; Shively, 1998b; Shively et al., 1997b). On the other hand, LHPA dysregulation in subordinate animals is typified by increased adrenal size (Shively and Kaplan, 1984; Shively, 1998a) and decreased glucocorticoid negative feedback following dexamethasone (Dex) injection (Collura et al., 2009; Jarrell et al., 2008; Kaplan et al., 2010; Shively, 1998b; Shively et al., 1997a; Wilson et al., 2008).

It is possible, however, that decreased glucocorticoid feedback in subordinate females can be explained apart from any central or pituitary dysregulation affecting LHPA activity. For example, metabolic clearance of Dex may be slower in dominant females, resulting in enhanced exposure and greater feedback inhibition. In addition, because decreased glucocorticoid negative feedback has been related to activity of estradiol in subordinate female macaques (Wilson et al., 2005), and because sex steroids can modulate adrenal morphology and function (Kasprzak et al., 1986; Malendowicz, 1986; Malendowicz et al., 1982) and the diurnal release of cortisol (Smith and Norman, 1987a), the use of cycling female macaques in previous studies comparing feedback inhibition, diurnal and ACTH-induced cortisol release between dominant and subordinate females (Riddick et al., 2009; Shively, 1998b; Shively et al., 1997b) may have contributed to variable results in tests measuring both of these parameters.

To address these potential confounds, we measured morning cortisol, glucocorticoid feedback inhibition as well as ACTH-stimulation of cortisol release, and Dex metabolism in ovariectomized (OVX), socially-housed female rhesus macaques. We

hypothesize that subordinate females will show altered LHPA responses compared to dominant females that will substantiate the contention that chronic social stress leads to alterations in the regulation of the LHPA axis in this primate species.

2.3 Materials and methods

Subjects. Subjects were long-term ovariectomized adult female rhesus monkeys (n=40) housed in small social groups consisting of six animals each (5 females and 1 male) at the Yerkes National Primate Research Center (YNPRC) Field Station. Behavioral data using an established ethogram (Jarrell et al., 2008) were collected for 30 min twice weekly during the 6 weeks of the initial phase of the study. Observational sessions were done between 1300 and 1400 hr. The total number of subjects classified in this fashion was 16 dominant and 24 subordinate females.

Morning basal cortisol and Dexamethasone Suppression Test (DST) Protocol. Subjects received Dex either at a dose of 0.125 mg/kg, IM or 0.25 mg/kg, IM in a counterbalanced manner separated by a one-month interval. Serum samples were collected at 0800, 1100, and 1730 hr. Morning baseline cortisol was accessed from the 0800 hr samples. Immediately following the sample at 1730 hr, females received the Dex injection. Samples were collected the following morning at 0800 and 1100 hr for the analysis of serum cortisol and Dex.

Adrenal response to ACTH. Following a previously described protocol (Shively, 1998b), subjects received an injection of dexamethasone (0.5 mg/kg, IM) at 0900 immediately following the collection of baseline plasma sample. Four hours later, a second serum sample was obtained followed immediately by an IV bolus of ACTH (10

ng/kg). Additional samples were collected 15 and 30 minutes following ACTH administration for cortisol assay.

Statistical analyses. Data were summarized as mean ± standard error of the mean (SEM). The main effects of status (dominant vs. subordinate), time before and after both doses of Dex, as well as their interactions were analyzed with analysis of variance for repeated measures. Preliminary analysis showed that 5HTT genotype did not influence LHPA activity as assessed in this study and thus 5HTT was consequently dropped from the analyses. A similar repeated measures approach was used for the analysis of the response to ACTH administration. Changes in cortisol levels due to Dex administration (pre- to post-Dex administration) as well as the area under the cortisol curve following ACTH administration were analyzed in a similar manner. When interactions or main effect of time were significant, Fisher post-hoc tests were completed, with appropriate corrections for multiple comparisons. In addition to using social status categories in the repeated measures ANOVA, Pearson product moment correlations were calculated between social rank (1 – 5), behavioral frequencies, and outcome measures from the Dex and ACTH tests. Stepwise multiple linear regression models were used to determine significant predictors for the suppression in serum cortisol by Dex as well as the area under the curve for the increase in serum cortisol following ACTH administration. Finally, boxplots were included showing variance in both of these outcome measures as a function of social rank. A test result with a $p \leq 0.05$ was considered significant

2.4 Results

Agonistic behavior varies by Social Status. Figure 2.1 shows rates of aggression received and submissive behavior emitted for monkeys at each social dominance rank

position. These data reflect agonistic behavior taken at six 30-minute observations throughout the study assessments. As expected, subordinate females received significantly more aggression ($F_{4, 35} = 8.17$, $p<0.001$) and emitted more submissive behaviors ($F_{4, 35} = 4.11$, $p=0.008$) (Figure 2.1A). Categorizing females ranked 1 and 2 as dominant and those ranked 3 through 5 as subordinate results in a significant main effect of social status for submissive behaviors emitted ($F_{1,38} = 9.73$, $p=0.003$) and aggression received ($F_{1, 38} = 11.9$, $p=0.001$) (Figure 2.1B).

Basal cortisol secretion. Early morning (0800 hr), mid-day (1100 hr) and late afternoon (1730 hr) cortisol levels were collected twice separated by a one-month interval as a part of the Dex suppression test (Table 2.1). Because these represent two samples of cortisol at each time of day, samples were averaged for analysis. As can be seen in Figure 2.2, early morning cortisol was significantly higher in dominant compared to subordinate females ($p = 0.032$). Serum cortisol did not differ between dominant and subordinate females at 1100 ($p = 0.99$) or 1730 hr ($p = 0.46$), reflecting a blunting of the diurnal rhythm in serum cortisol in subordinates.

Glucocorticoid negative feedback. Serum cortisol levels varied significantly over time with Dex administration (Table 2.1; $F_{1, 38} = 8.42$, $p = 0.006$), as cortisol levels at 0800 and 1100 following Dex administration were significantly lower than baseline levels of cortisol at 0800 ($p < 0.001$) and 1100 ($p < 0.001$) before the Dex was administered (Table 2.1). This main effect of time interacted significantly with dose of Dex administered ($F_{1, 38} = 8.42$, $p=0.006$). Whereas cortisol values were not significantly different between the low (4.71 ± 1.21 µg/dl) and high dose (4.00 ± 0.54 µg/dl, $p = 0.42$) at the 0800 time following Dex, cortisol levels at 1100 post-Dex were significantly higher

following the low dose (6.08 ± 0.92 µg/dl) compared to the high dose (4.11 ± 0.51 µg/dl; p = 0.004).

There was a significant status by time from Dex interaction ($F_{1, 38} = 4.96$, p = 0.032) that was not influenced by dose (p = 0.641), as serum cortisol was significantly higher in subordinates (5.12 ± 0.79 µg/dl) compared with dominant females (3.51 ± 0.99 µg/dl) following Dex but, as described above, baseline or early morning cortisol was higher in dominant females (25.04 ± 1.14 vs. 23.407 ± 0.91 µg/dl). Consequently, the suppression in cortisol due to Dex administration was determined by calculating the change in cortisol from the pre-Dex to post-Dex levels at both 0800 and 1100 for both doses of Dex. There was a main effect of status, as suppression of cortisol by Dex was significantly less in subordinates (-18.29 ± 0.82 µg/dl) compared with dominant females (-21.50 ± 1.04 µg/dl; $F_{1, 38} = 5.92$, p = 0.020). The high dose of Dex induced a significantly greater change in serum cortisol (-21.53 ± 0.84 µg/dl) compared with the low dose (-18.25 ± 0.90 µg/dl; $F_{1, 38} = 8.42$, p=0.004) and this effect was similar for subordinate vs. dominant animals (no dose by status interaction; p = 0.82). However, this effect of status did vary significantly over time from Dex ($F_{1, 38} = 4.96$, p = 0.032). The change in serum cortisol was significant greater at 0800 following either dose of Dex in dominant (-24.54 ± 1.42 µg/dl) compared with subordinates (-18.78 ± 1.12 µg/dl; $F_{1, 38} = 10.01$, p=0.003), an effect that disappeared by 1100 (p = 0.95; Figure 2.3).

Multiple regression analyses were performed to determine how status and serum levels of Dex resulting from the injections predicted serum concentrations of cortisol. For the low dose Dex, the change in serum cortisol at 0800 hr was significantly predicted by social status (R = 0.34, p = 0.03) while serum Dex failed to significantly improve

predictability of the model ($p = 0.11$). Similarly, for the high dose Dex, the change in serum cortisol at 0800 hr was significantly predicted by social status ($R = 0.44$, $p = 0.03$) while serum Dex also failed to significantly improve predictability of the model ($p = 0.83$). Neither social status nor serum Dex significantly accounted for variance in the change in serum cortisol at 1100 hr following the low or high dose Dex.

Adrenal responsivity to ACTH. Serum cortisol varied significantly over time during the ACTH stimulation test with highest values at 0800 prior to Dex suppression and nadir values 4 hour later prior to ACTH administration ($F_{3, 114} = 81.09$; $p < 0.001$). Subsequent to the ACTH injection, cortisol levels peaked at +15 min (Figure 4A). Importantly, time interacted with social status ($F_{3, 114} = 2.57$, $p = 0.05$) to influence cortisol responsivity as dominant animals showed greater elevations in cortisol levels following ACTH administration at +15 ($p = 0.021$) compared to subordinate females (Figure 2.4A). The analysis of area under the curve (AUC) of cortisol levels showed that dominant animals had overall increased cortisol levels in response to the ACTH compared to subordinate females (Figure 2.4B; $F_{1, 38} = 5.86$, $p=0.020$).

Behavioral predictors of cortisol responsivity. Table 2.2 shows Pearson product moment correlations between social rank (1 – 5), behavioral frequencies, and outcome measures of Dex suppression (change in serum cortisol at 0800 hr) and ACTH stimulation (AUC). As expected, social rank was significantly related to aggression received ($p < 0.001$) from others and submissive behavior directed to others ($p = 0.001$). Aggression directed towards others was not related to rank, indicating that the highest-ranking females are not necessary the most aggressive. Importantly, there was a significant negative correlation between rank and Dex suppression of serum cortisol ($p =$

0.046) and ACTH stimulation of serum cortisol ($p = 0.015$), indicating these responses decrease with increasing subordination. Furthermore, animals that show low rates of submissive behavior responded with a greater decrease in cortisol following Dex ($p = 0.008$). In addition, the increase in cortisol following ACTH was positively related to the amount of aggression directed to others ($p = 0.026$) and negatively related to aggression received from others ($p = 0.027$) and submissive behaviors emitted ($p = 0.030$). Finally, there was also a significant negative correlation between Dex suppression and ACTH stimulation (Figure 5; $p = 0.007$), indicating that animals showing a reduced sensitivity to Dex suppression also showed reduced increases in response to ACTH.

Stepwise multiple regression analyses were performed to determine how social rank and behavioral frequencies predicted the change in serum cortisol following Dex and the AUC for cortisol following ACTH stimulation. With respect to the average change in serum cortisol at 0800 hr following the low and high dose of Dex, only rates of submissive behavior significantly predicted serum cortisol ($R = 0.42$ $p = 0.008$). No other predictors were entered into the equation because they did not account for any additional variance in serum cortisol. A similar analysis of the AUC for serum cortisol following ACTH stimulation revealed that only social rank significantly predicted serum cortisol ($R = 0.38$, $p = 0.015$). No other predictors were entered into the equation.

2.5 Discussion

Results here show that social subordination in female rhesus monkeys leads to a disruption in feedback inhibition of the LHPA axis that is not due to differences in serum dexamethasone following treatment and occurs in the absence of ovarian hormones. In

addition, results suggest that adrenal responsivity is reduced in subordinate females resulting in a dampened cortisol response to ACTH. This reduced adrenal sensitivity may also contribute to the blunted diurnal rhythm of cortisol observed in subordinate females. These differences in LHPA responsivity were similar whether status categories of dominant versus subordinate are used or whether linear ranks are used. These data suggest that chronic exposure to psychosocial stress in female rhesus macaques, induced by rank-related differences in agonistic behaviors, produces a distinct LHPA phenotype in which the ability to both mobilize and curtail glucocorticoids is significantly compromised.

Previous studies indicate that social subordination in macaques (Collura et al., 2009; Jarrell et al., 2008; Kaplan et al., 2010; Shively, 1998b; Shively et al., 1997b) and chronic exposure to stressors in rodents (Young and Korszun, 1998) decreases glucocorticoid negative feedback similar to humans suffering from depression and other psychopathologies (Holsboer, 2001; Kalin et al., 1982; Raison and Miller, 2003). Rodents subjected to chronic stress often show increased basal glucocorticoid level; however, this is found only in a subset of human beings suffering from psychiatric disorders (Capuron et al., 2003). Dysregulation of glucocorticoid receptor (GR) signaling, including reduced glucocorticoid negative feedback, is more consistently implicated in human psychopathology (Raison and Miller, 2003). Diminished negative feedback inhibition of the LHPA axis in rodents is due to decreases of GR and mineralocorticoid receptors in the hippocampus and the hypothalamus (Aguilera and Rabadan-Diehl, 2000; Bhatnagar and Dallman, 1998; Kovacs et al., 2000). In monkeys, this resistance to dexamethasone suppression likely reflects a decrease in limbic and pituitary GR expression (Brooke et

al., 1994). This is also suggested to be the case in humans (Pariante and Miller, 2001). In rats, reduced glucocorticoid sensitivity that is associated with chronic exposure to stressors is linked to increases of corticotropin-releasing hormone (CRH) in the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Albeck et al., 1997; Keen-Rhinehart et al., 2009; Stout et al., 2000) and increases arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (PVN) (Ma et al., 1999; Makino et al., 1995). Analysis of CRH and AVP in the cerebral spinal fluid from dominant and subordinate female macaques is planned to examine if these neuropeptides are also altered by chronic psychosocial stress.

In this study dominant females had significantly higher morning basal cortisol levels than subordinates. Consistent differences in basal morning cortisol due to social status in macaques species has not been reported in the literature thus far (Czoty et al., 2009; Gust et al., 1993; Stavisky et al., 2001). It is possible these discrepancies are a result of the small sample size usual to studies in non-human primates. The effect of sex hormones in the control of diurnal cortisol secretion may be another factor. A previous study examining the effect of ovariectomy on circadian cortisol in female macaques found that absolute levels of cortisol were lower in OVX females, but there was no disruption of the circadian rhythm of cortisol (Smith and Norman, 1987b). However, female monkeys in this investigation showed a rise in mid-day cortisol that may have been caused by the feeding or room-cleaning schedule that took place regularly at 12 p.m. each day. The rise in cortisol at mid-day may have activated negative–feedback mechanisms that would have caused a decline in evening levels of cortisol and masked accurate detection of basal cortisol. Studies with OVX rats replaced with estrogen found

that the evening elevation in corticosterone was significantly enhanced by estrogen, and estrogen treatment reversed the blunting of the diurnal rise in corticosterone by Dex injection (Weiser and Handa, 2009). Hence, it may be that using a relatively large number of monkeys here, and/or by using only OVX females so as to preclude differences due to sampling females who are at different points in their ovarian cycles, unmasked the blunted morning cortisol release occurring in subordinate female monkeys.

The cortisol response to ACTH administration during an ACTH challenge has also been inconsistent in differentiating dominant from subordinate macaques (Riddick et al., 2009; Shively, 1998b; Shively et al., 1997b). In this study dominant animals had increased cortisol levels in response to the ACTH compared to subordinate animals. Thus, despite the adrenal enlargement that is observed in necroscopic studies from subordinate macaques (Shively and Kaplan, 1984) and which we are assuming is the case in the subordinate females in this study, these females did not have enhanced cortisol release following ACTH. Chronic stress produces enlargement of the adrenal gland and hypercortisolemia in rats (Blanchard et al., 1993; Spencer and McEwen, 1990; Tache et al., 1978). Enlarged adrenals concomitant with increased secretion of cortisol are present in some populations of people with clinical depression (Nemeroff et al., 1992; Rubin and Phillips, 1993; Rubin et al., 1995; Stokes, 1995), and are thought to be predictive of ensuing depression for people undergoing stressful life events (Ising et al., 2005). However, studies of chronic stress effects on adrenal structure and function in laboratory rats are limited in duration and thus are probably not producing the same stage of chronic stress exposure as is present in our subordinate monkeys. One study in rodents that disambiguated adrenal reactivity (that is increased ACTH-stimulated corticosterone

release due to enlargement of tissue and enhanced out-put) from increased sensitivity (increased ACTH-stimulated corticosterone due to increased adrenal cortical response to ACTH) suggested that 14 days of chronic variable stress increased adrenal reactivity and not sensitivity (Ulrich-Lai et al., 2006). Again, 14 days of stress, however intense, is unlikely to duplicate the months of chronic psychogenic stress experienced by subordinate female macaques living in groups for years. Indeed, we speculate that a similar change in adrenal size and output is occurring in the first weeks following the onset of social subordination in monkeys and in the clinical studies on stressed people mentioned above. However, we believe that a more probable explanation for enlarged adrenals concurrent with reduced ATCH stimulated cortisol as seen in this present study is adrenal exhaustion. *In vitro* studies on extracted adrenal cortical tissue have shown that constant application of ACTH first causes tissue hypertrophy and increased glucocorticoid release followed by a drastic reduction in glucocorticoid secretion (Lamberts et al., 1987).

Previous studies in cynomolgus macaques assessing adrenal response to ACTH administration have shown either increased cortisol response to ACTH (Czoty et al., 2009) in subordinate males compared to dominant male monkeys or no social status differences in this LHPA measure in female monkeys (Riddick et al., 2009). While species differences could account for the differences in ACTH response, the simplest explanation is that in these studies, LHPA activity is modulated by hormonal status, as has been seen in rodents (Viau, 2002; Viau et al., 2005; Viau et al., 2001), monkeys (Stavisky et al., 2003; Wilson et al., 2005; Wood et al., 2004), and women (Burleson et al., 1998; Gudmundsson et al., 1999). Testosterone and estrogen have been shown to

affect adrenal sensitivity to ACTH in male and female rats (Handa et al., 1994b; Nowak et al., 1995; Sapolsky et al., 1983) as well as in men and women (Goel and Bale; Kudielka and Kirschbaum, 2005; Young). The study by Riddick and colleagues cited above (Riddick et al., 2009) tested adrenal responses to ACTH during several days of the follicular phase in ovarian intact female cynomolgus monkeys. Therefore, the differences in ACTH induced cortisol between this investigation and that prior study by could be specifically due to sampling affected by individual differences in changing levels of sex steroids that modulated the responsivity of the adrenal gland.

It is important to note that the current study did not include a controlled injection of CRH. Such a treatment would act on the pituitary to stimulate ACTH release and would have revealed differences in pituitary corticotrope sensitivity to due to social status. Changes in the responsivity of the pituitary due to chronic stress (De Goeij et al., 1992a; De Goeij et al., 1992b; Lee et al., 2011) and reproductive stage (Toufexis et al., 1999) have been suggested to occur in rodents, and in war veterans with PTSD (Golier et al., 2011). Similar changes may take place due to chronic psychogenic stress in subordinate monkeys. Future studies are planned to determine if changes in pituitary responsivity to CRF and/or AVP exist in subordinate female monkeys.

The primary approach used in this present study was to quantify LHPA responsivity in females from multiple 5-member groups, categorizing dominants as females ranked 1 and 2 and subordinates as those ranked 3 – 5. This convention has been used for nearly 30 years to assess dominance status effects on a vast number of outcome measures in captive macaques (e.g., (Kaplan, 2008; Kaplan et al., 1995; Kaplan et al., 1984; Kaplan et al., 2010; Kaplan et al., 1982; Kaplan et al., 2002; Michopoulos et al.,

2010; Paiardini et al., 2009; Shively and Kaplan, 1984; Shively, 1998b; Shively and Clarkson, 1994; Shively et al., 1997a; Shively et al., 1997b), an approach similar to the analysis of social stress effects in people (Marmot, 2006). The accepted rationale for this approach is to increase statistical power by comparing females who receive limited aggression and must submit less to those that receive proportionately more aggression and more frequently terminate these interactions by submitting more. While this approach of categorizing females as dominant or subordinate could be seen as a limitation compared to an analysis using linear ranks of the females, the correlation analyses using rank were consistent with the results of the status categories using ANOVA. Social rank (1 – 5) showed a significant negative correlation to the suppression in serum cortisol following dexamethasone, indicating that with increasing dominance rank, serum cortisol is suppressed more. In addition, higher rank also was related to a larger increase in cortisol following ACTH stimulation. Examination of the boxplots for both the response to dexamethasone and ACTH (Figure 2.6) shows a more rank-related gradient for the response to ACTH whereas the response to dexamethasone for females ranked 1 and 2 was similar yet distinct from those ranked 3 – 5. The biological mechanism accounting for these two patterns of stress hormone responsivity are unexplored, but the data imply there is not a gradient in the loss of GR with increasing subordinate status.

In addition, the correlational analysis shows two key behaviors defining subordinate status, aggression received from others and submission emitted, are highly predicted from rank but not perfectly, suggesting, for example, that the lowest ranking female does not always received the most aggression nor are the most submissive in

terms of frequency of behavior. As long as a female submits to every other monkey in her group, she would be classified as the most subordinate. However, the regression analysis showed that submissive behavior was the best predictor of the efficacy of Dex suppression. Because submissive behavior is emitted in response to perceived threats, it likely is a strong reflection of the number of needed attempts to terminate potentially harmful interactions. While the use assessment of specific rank related differences in LHPA regulatory mechanisms would be informative, the present data nonetheless shows distinct phenotypes between more dominant compared with more subordinate females.

Overall, our results here suggest that social subordination in female rhesus monkeys results in a diminished adrenal response to circadian- or ACTH-induced stimulation, along with impaired inhibitory control following LHPA axis activation. Compilations of studies in animals and humans have led to the conclusion that there is an optimum level of LHPA functionality that exists to deal with the metabolic and behavioral requirements of stress in the most advantageous fashion. Consequently, under or over activity of the LHPA system are both suboptimal and increase allostatic load on the organism and the propensity for dysregulation (for review see:(Chrousos, 2009)). Dominant female monkeys here display a finely tuned LHPA system allowing an ample response to stress as well as efficient inhibition subsequent to activation. Importantly, dominant monkeys retain the ability to mount a robust cortisol response following dexamethasone suppression. In fact, the degree of suppression after Dex predicts the subsequent response to ACTH stimulation, further underscoring the flexibility of LHPA reactivity in dominant animals. Subordinate females in the current study were unable to mount a similar response in either direction. Several human pathologies are related to

both under and over active LHPA responses (Chrousos, 2009). Those related to hypo-responsive LHPA activity include: adult post-traumatic stress disorder and seasonal affective disorder (Chrousos and Gold, 1992; Pervanidou and Chrousos), premenstrual syndrome (Chrousos et al., 1998), chronic fatigue syndrome (Clauw and Chrousos, 1997), postpartum depression (Magiakou et al., 1996), fibromyalgia (Clauw and Chrousos, 1997), and perimenopausal depression (Chrousos et al., 1998). The particular deficits in the LHPA system observed in our subordinate female monkeys may be particularly relevant to the study of these human disorders. Indeed, considering the relationship that is believed to exist between stress and human illness (McEwen, 2000), many physiological as well as psychological similarities may exist between human beings and macaques given that psychosocial stress in these monkeys produces a related type of LHPA dysregulation.

Table 2.1. Mean ± SEM levels of serum cortisol prior to and following two doses of Dex (Low 0.125 and High 0.25 mg/kg) as a part of a DST. Asterisks denote significantly lower levels of cortisol following administration of Dex compared to baseline values prior to Dex.

Time	0800	1100	1730	0800	1100
Low Dose					
Dominant	26.42 ± 1.75	20.66 ± 1.53	19.2 ± 1.54	3.17 ± 1.95*	4.45 ± 1.14*
Subordinate	23.85 ± 1.37	21.62 ± 1.21	19.1 ± 1.36	5.74 ± 1.59*	6.17 ± 1.16*
High Dose					
Dominant	29.02 ± 1.55	24.07 ± 1.84	24.9 ± 1.67	3.19 ± 0.87*	3.35 ± 0.67*
Subordinate	23.96 ± 1.22	24.20 ± 1.50	22.3 ± 1.34	4.51 ± 1.07*	4.07 ± 0.66*

Table 2.2. Pearson product moment correlations between social status ranks (1 – 5), measures of the change in serum cortisol in response to dexamethasone suppression (Dex) and ACTH stimulation, and rates (per hr) of social and anxiety behaviors. *p < 0.05; ** p < 0.01.

Variable	**Rank**	**Dex**	**ACTH**
Rank	-		
Dex suppression	-0.33*	-	
ACTH stimulation	**-0.38***	**-0.43****	-
Aggression - actor	-0.17	-0.06	**0.35***
Aggression - recipient	**0.60****	0.19	**-0.35***
Affiliation - actor	-0.27	-0.30	0.15
Affiliation - recipient	0.26	0.09	-0.02
Submission - actor	**0.52****	**0.42****	**-0.34***
Anxiety	-0.05	0.02	-0.21

Figure 2.1. (A) Rates (per 30 min) of aggressive behavior received and submission behavior emitted by females at each social dominance rank. Rates of behavior were averaged across the 12 observational sessions for a particular female. Rates were then averaged across females to generate the mean ± sem rate for a specific rank. (B) Mean ± sem rates of aggression received and submission towards others in females categorized as dominant rank 1 and 2, and subordinate (ranks 3 – 5). Rates of aggression received ($p = 0.001$) and submission emitted ($p = 0.003$) were higher in animals categorized as subordinate females (ranks 3 – 5) compared with those categorized as dominant (ranks 1 - 2).

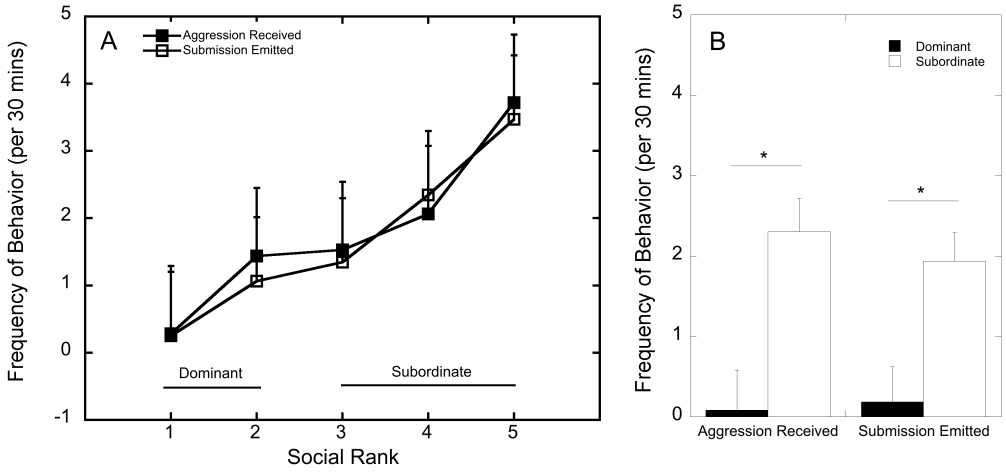


Figure 2.2. Mean ± SEM serum concentrations of cortisol at 0800, 1100, and 1750 hr for dominant and subordinate females. Values represent the average of the two sampling periods prior to the low and high dose Dex challenge test. The asterisk indicates dominant females were significantly different than subordinates ($p < 0.05$).

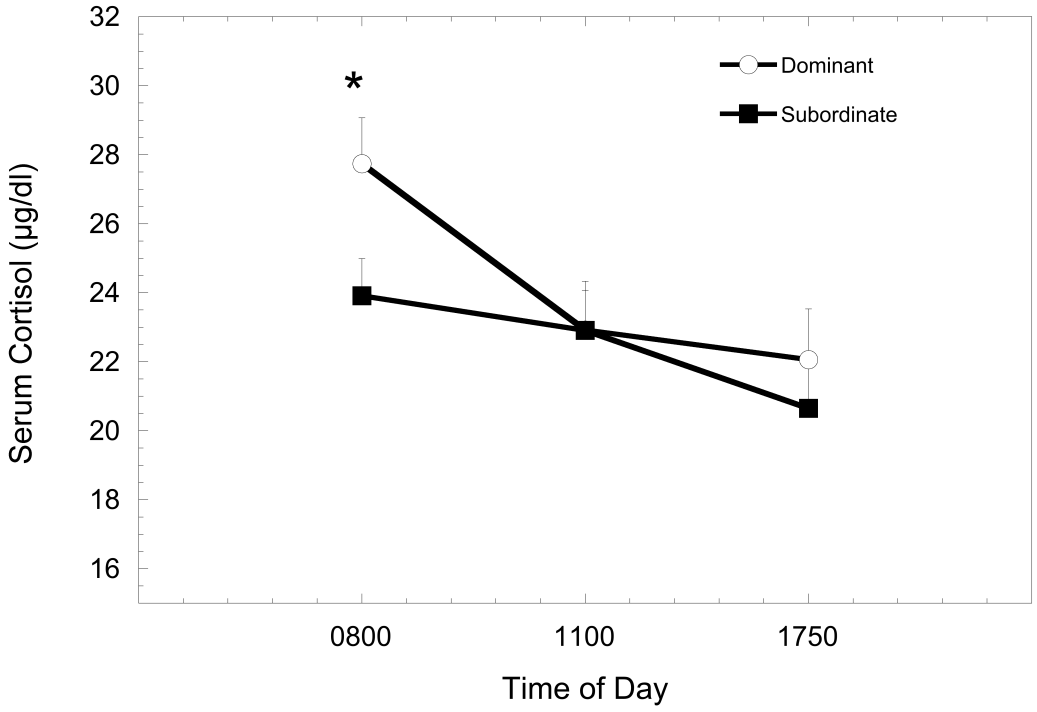


Figure 2.3. Mean ± SEM change of serum cortisol at 0800 and 1100 hr following low (0.125 mg/kg) and high dose Dex (0.25 mg/kg) administered at 1750 hr the evening before for dominant and subordinate females. Asterisk denotes significantly greater decrease in cortisol at both doses in dominant compared to subordinate females ($p < 0.05$).

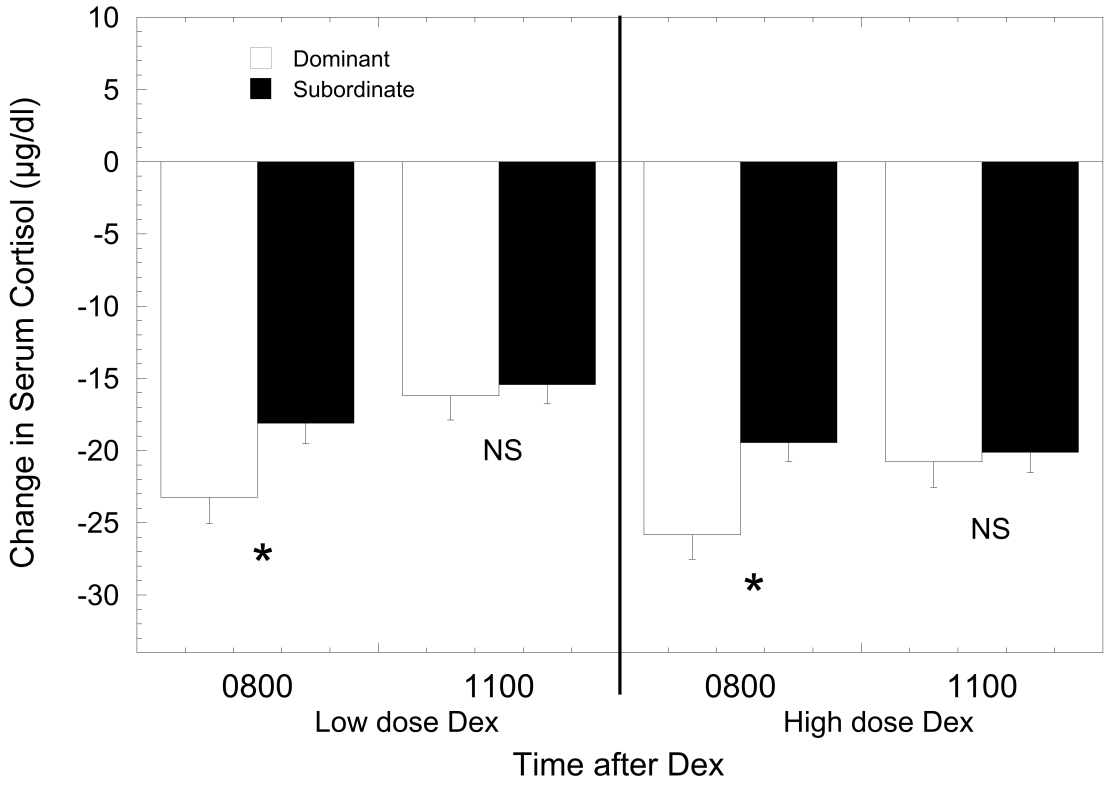


Figure 2.4. (A) Mean ± SEM levels of serum cortisol during the ACTH stimulation test for dominant and subordinate females. Dex (0.50 mg/kg) was administered following the sample at 0800 hr and ACTH (10 ng/kg) following the sample at 1200 hr. (B) Mean ± SEM values for the area under the curve of cortisol levels following ACTH administration. Asterisks denote significant status differences in cortisol levels.

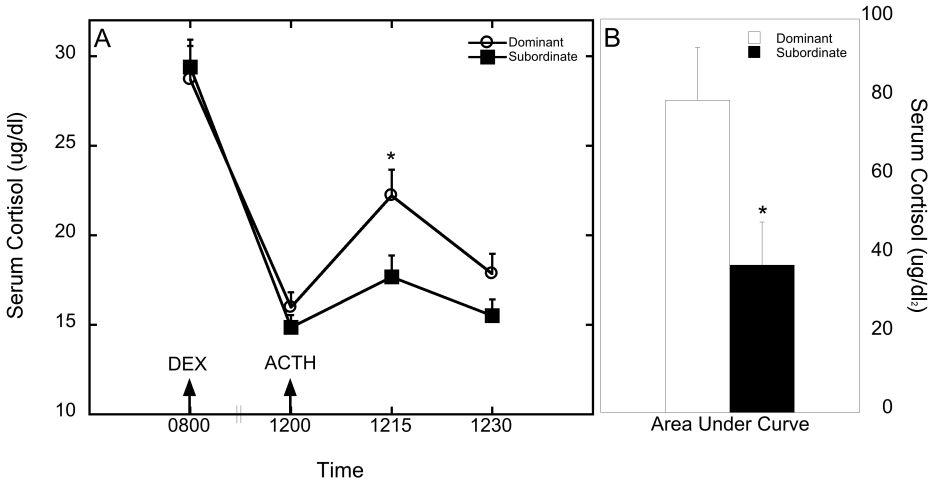


Figure 2.5. Pearson product moment correlation between the cortisol area under the curve response to ACTH administration and the mean change in serum cortisol at 0800 hr following Dex suppression (averaged across low and high dose of Dex).

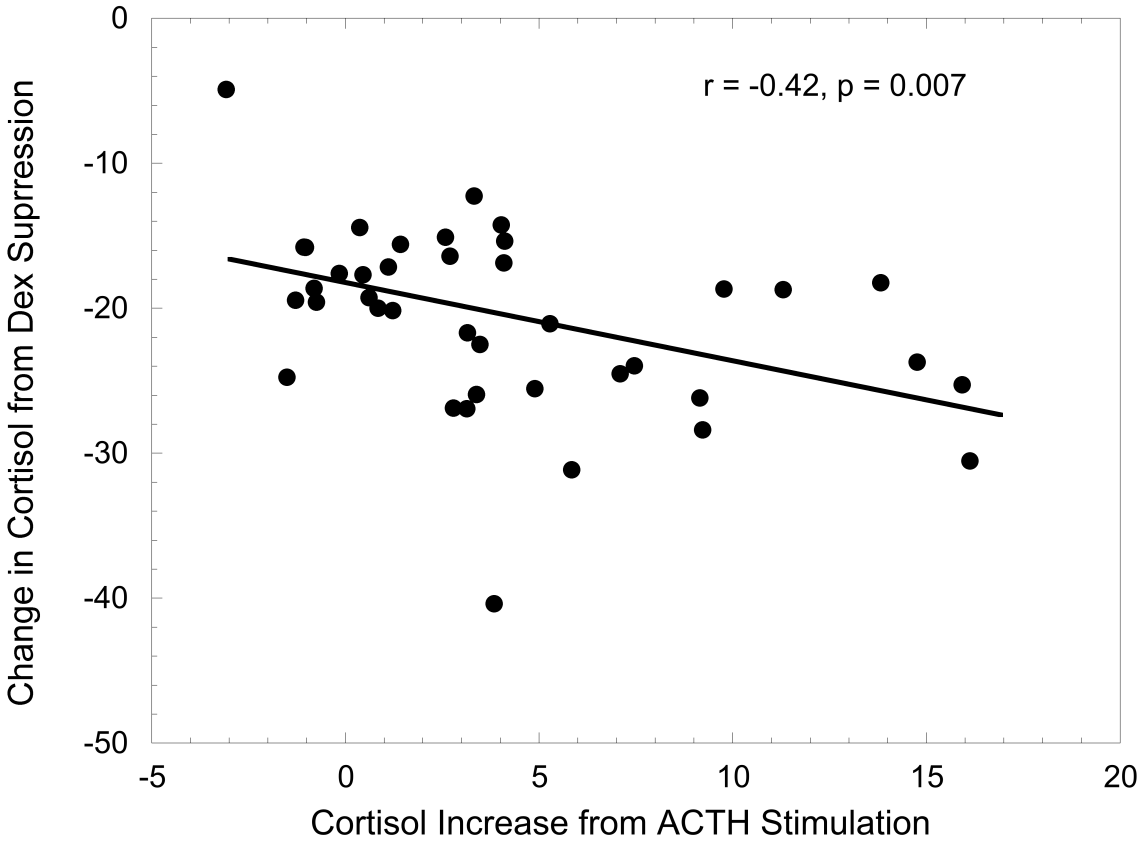
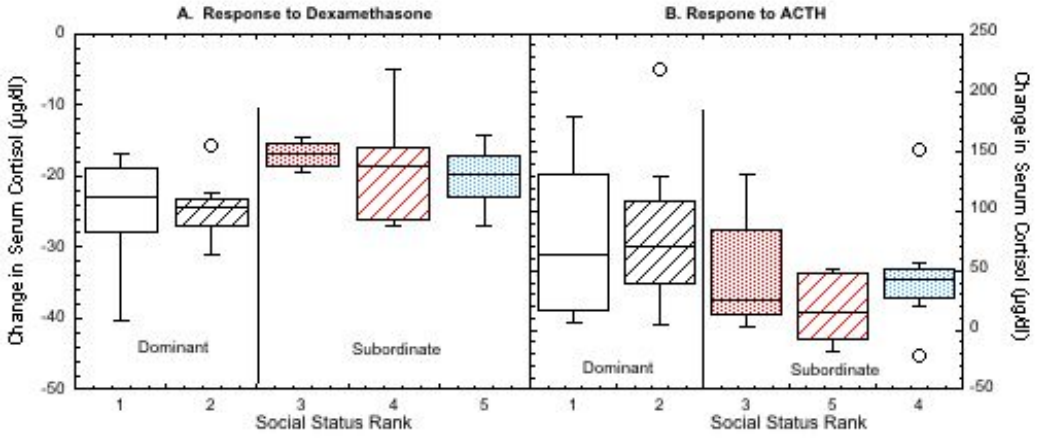


Figure 2.6. Boxplots of the change in serum cortisol at 0800 hr following Dex suppression (A) and the area under the cortisol response curve following ACTH administration (B) by social rank (1 – 5).



CHAPTER THREE:

SOCIAL SUBORDINATION ALTERS SOCIOEMOTIONAL EFFECTS OF ESTRADIOL IN FEMALE RHESUS MONKEYS

[*All text and figures from (Michopoulos et al., 2011b)*]

3.1 Abstract

Despite the well-documented relation between estradiol (E2) and behavior, exposure to stressors may modify sensitivity to E2. The effects of E2 on behavior are, in part, likely related to their modulation of the serotonin (5HT) and oxytocin systems. The short allele (s-variant) polymorphism found in the promoter region of the *SLC6A4* gene that encodes the 5HT transporter (5HTT) modulates responsivity to stressors. The current study used ovariectomized adult female rhesus monkeys to evaluate how exposure to the psychosocial stressor of social subordination and polymorphisms in the gene encoding 5HTT influence the behavioral effects of E2 and immunoreactive serum oxytocin. Dominant females had higher levels of oxytocin than subordinate animals even though E2 increased immunoreactive serum oxytocin in all females. E2 increased affiliative behaviors in all animals, with even more of these prosocial behaviors directed at dominant females. S-variant females, regardless of social status, were more aggressive towards more subordinate cage mates and these behaviors too were increased by E2. Subordinate s-variant females are most often involved in agonistic behavior, less affiliative behavior, and were less responsive to the anxiolytic action of E2. The results show that the short allele of the 5HTT gene synergizes with psychosocial stress exposure to affect the behavioral efficacy of E2 while confirming the actions of E2 for producing generalized behavioral arousal in females. Whether differences in the central action of 5HT and/or oxytocin are responsible for this effect requires further study.

3.2 Introduction

Estradiol (E2) is a pleiotropic hormone that targets multiple neurochemical systems regulating a range of behaviors (McEwen, 2002; Pfaff et al., 2000) that likely enhances a female's ability to attend to contextual demands of their environment (Morgan et al., 2004). Exposure to increased concentrations of E2 is generally accepted to be critical for the expression of female sexually motivated behavior in a number of animal models (Blaustein et al., 1987; Pope et al., 1987; Rissman et al., 1997b; Wallen and Tannenbaum, 1997) as well as in women (Dennerstein et al., 1980). Despite this relation between E2 and behavior, exposure to stressors or stress hormones appears to disrupt the behavioral effects of E2. Restraint (Uphouse et al., 2005) or psychosocial stress (Pierce et al., 2008a), as well as overexpression of corticotropin releasing-hormone (CRH) in the central nucleus of the amygdala (Keen-Rhinehart et al., 2009), reduces sexual behaviors in ovariectomized, hormone-primed rodent females, an effect that is overcome by higher doses of hormones (White and Uphouse, 2004). Imposition of subordination accomplished through harassment and noncontact aggression in macaque species is considered a potent psychosocial stressor (Sapolsky, 2005), as subordinate animals are hypercortisolemic due to diminished glucocorticoid negative feedback (Kaplan et al., 1984; Shively, 1998b; Wilson et al., 2008). In this context, proceptive behavior occurs more frequently in dominant compared to subordinate females (Shively et al., 1990b) and at lower concentrations of circulating E2 (Wallen, 1990). While the consequences of psychosocial stress on E2-dependent changes in sexual behavior appear robust, it is unclear whether stressed-induced changes in signals from the limbic-

hypothalamic-pituitary-adrenal (LHPA) axis affects the efficacy of E2 on other aspects of socio-emotional behaviors, including affiliation, aggression and anxiety-like behavior.

The effects of E2 on social behaviors are, in part, likely related to their modulation of the serotonin (5HT) neural system by increasing 5HT synthesis and modulating 5HT reuptake transporter (Bethea et al., 2002). The observations that stress or CRH administration reduces 5HT in the median raphe (Summers et al., 2003; Umriukhin et al., 2002) and decreases 5HT release to limbic structures (Price and Lucki, 2001; Thomas et al., 2003) provides a possible neuroanatomical support for the hypothesis that stress impairs E2 facilitation on behavior. Furthermore, reduced transcriptional activity of the *SLC6A4* gene encoding the 5HT transporter (5HTT) due to naturally occurring polymorphisms in the length of the promoter region may also disrupt E2 action as the short promoter length (s-variant) of the *SLC6A4* gene is associated with a range of behavioral phenotypes, including increased anxiety, aggression, and impulsivity in humans (see (Murphy and Lesch, 2008)). These length variations with reduced transcriptional activity are also present in rhesus monkeys and the response to an acute psychosocial stressor appears greater in animals with a s-variant promoter length allele (l/s or s/s) compared to those with long promoter length alleles (*l/l* genotype) (Bennett et al., 2002; Lesch et al., 1997). It is not clear whether females with the s-variant of the 5HTT gene are less responsive to the behavioral effects of E2 and whether this is worsened by social subordination.

In addition to 5HT, another potential target of E2 in the brain for the prosocial and anxiolytic effects of E2 is oxytocin (Insel et al., 1998; Windle et al., 1997). In rodent models, E2 upregulates the expression of oxytocin (Lim and Young, 2006) and the

oxytocin receptor (Choleris et al., 2003; Patisaul et al., 2003) and increases oxytocin binding density in limbic regions (McCarthy et al., 1996). While oxytocin may be important for attenuating hormonal markers of stress (Neumann et al., 2000; Nomura et al., 2003), chronic stressor exposure or corticosterone administration upregulates hypothalamic oxytocin levels (Laguna-Abreu et al., 2005; Paredes et al., 2006) and oxytocin receptor binding (Liberzon and Young, 1997) in rodents. These data would imply that the oxytocin system might be unregulated in socially subordinate females; however, it is not known how E2 would affect oxytocin activity in females exposed to different amounts of psychosocial stress.

The present study used ovariectomized adult female rhesus monkeys to determine how social subordination influences the behavioral effects of E2 and whether these effects were modified by 5HTT genotype. The study was designed to test the hypothesis that the prosocial behavioral effects of E2 would be diminished in subordinate females, particularly those with the s-variant allele in the gene encoding the 5HTT, and these differences may be associated with differences in oxytocin. Because evidence suggests that s-variant animals show more impulsive and aggressive behavior, we predicted that E2 would increase aggression significantly more in females with the s-variant 5HTT genotype. Finally, in order to test the hypothesis that differences in stress hormone action accounted for these status differences in the behavioral effects of E2, we predicted that administration of a CRH receptor antagonist would increase the prosocial effects of E2 in subordinate females.

3.3 Material and methods

Subjects. Subjects were 37 ovariectomized adult female rhesus monkeys that were housed in one of eight small social groups at the YNPRC Field Station (n = 4 or 5 per group) without adult males. Of the total 37 subjects, the genotype and status distributions were: 8 s-variant dominant, 12 s-variant subordinate, 8 l/l dominant, and 9 l/l subordinate. Groups had been formed for 18 months prior to the start of the present study and were ovariectomized 6 months prior to the formation of these groups.

Experimental design. All animals were studied in each of four treatment conditions that each lasted one week and were separated by a two-week, no treatment washout period. The order of treatments was counterbalanced across groups, with all females in a specific social group receiving the same treatment. Specifically, the four treatments consisted of control (placebo), E2 replacement (E2), CRH receptor analogue (CRHA), and E2 plus CRHA. A 0.25 ml sc injection of saline was administered at 0830 hours for five consecutive days as the control condition. E2 replacement was accomplished by implanting E2 filled Silastic capsules sc as previously described (Mook et al., 2005). Analysis of selected samples (days 4 and 7) during E2 replacement versus no E2 replacement conditions indicated that hormone replacement achieved mid-follicular phase concentrations (66.7 ± 2.1 vs. < 5.0 pg/ml). Capsules were implanted three days prior to the initiation of data collection and removed immediately following the end of the phase.

The CRHA utilized for the study was a CRH type 1 receptor antagonist CP154,526 (Pfizer, Groton CT), an analogue of the more widely used antalarmin (Seymour et al., 2003). CP154,526 was used as it crosses the blood brain barrier to bind

to both peripheral and central CRH type 1 receptors (Seymour et al., 2003). Based on the existing literature in monkeys using antalarmin (Ayala et al., 2004; Broadbear et al., 2004; French et al., 2007; Habib et al., 2000), we chose to administer a dose of 10 mg/kg sc daily for five consecutive days at 0830 hours with the expectation that it would attenuate cortisol secretion. However, as described in ***Results*** and reported previously (Broadbear et al., 2004), this dose paradoxically stimulated cortisol secretion, allowing us to evaluate the impact of an increase in cortisol on behavior in female monkeys.

Outcome measures. The objective of this study was to assess how social subordination and 5HTT genotype may modify the effects of E2 on socio-emotional behavior. Behavioral data were collected using an established ethogram (Jarrell et al., 2008) by taking 30-minute group observations. These observations were done five hours following saline or CRHA injection on each day of injection. Affiliative behavior was comprised of proximity and grooming; aggression was defined by threats, slaps, grabs, and bites; and submissive behavior was characterized by withdrawals, grimaces, and screams. Anxiety-like behavior consisted of body shakes, yawns, self-scratching, and self-grooming (Troisi, 2002).

Serum samples were collected at 0900 hours on days 4 and 7 to confirm E2 concentrations. Samples were collect on days 4 – 7 for morning cortisol analyses. While it is thought that peripheral levels of oxytocin do not reflect central oxytocin (Neumann, 2007), recent neuroanatomical evidence indicates magnocellular oxytocin neurons from the paraventricular nucleus of the hypothalamus (PVN) project to both the posterior pituitary and forebrain structures, suggesting serum oxytocin could be surrogate markers of centrally active oxytocin (Ross and Young, 2009). Consequently, we measured

immunoreactive serum oxytocin on days 5 and 6 of each treatment condition to assess cumulative changes in peptide concentrations.

Statistical analysis. Data were summarized as mean ± standard error of the mean (SEM). In order to examine the categorical (status and genotype) and treatment (E2, CRHA), and day on treatment main effects and their interactions, data were analyzed with repeated measures analysis of variance in a 2 by 2 design to assess E2 (E2 and E2+CRHA) vs. non-E2 treatments (C and CRHA) and CRHA (CRHA and E2+CRHA) vs. non-CRHA treatments (C and E2). All statistical tests with $p \leq 0.05$ were considered significant and post-hoc corrections were made for multiple comparisons when necessary.

3.4 Results

Social status categorizations based on agonistic behavior. Figure 3.1 illustrates the mean frequency of aggression received and submissive behavior emitted across females at each rank collapsed across all treatment conditions and time. Data describing treatment effects on these behaviors is presented below. Females ranked 3 – 5 received significantly more aggression from higher-ranking group mates ($F_{4, 27} = 11.4$, $p < 0.001$). This harassment was associated with rank-dependent, higher rates of submissive behavior ($F_{4, 27} = 6.56$, $p < 0.001$).

Serum Cortisol. As illustrated in Table 3.1, E2 decreased ($F_{1, 33} = 14.90$, $p < 0.001$) and the CRHA increased ($F_{1,33} = 12.55$, $p < 0.01$) morning cortisol values in all subjects. Serum cortisol was consistently lower throughout the week of E2 treatment compared to placebo ($F_{3, 99} = 0.24$, $p = 0.87$) but did increase progressively throughout the week during the CRHA treatment (data not shown; $F_{3, 99} = 6.55$, $p < 0.01$). Overall

mean levels of cortisol were higher during CRHA even when combined with E2. Neither social status ($F_{1, 33} = 2.08$, $p = 0.16$), 5HTT genotype ($F_{1, 33} = 0.01$, $p = 0.96$) nor the interaction of status and genotype ($F_{1,33} < 0.01$, $p = 0.99$) significantly influenced the effect of E2 on cortisol concentrations. Similarly, the CRHA–induced elevation in cortisol was unaffected by status ($F_{3,99}=0.96$, $p=0.41$), genotype ($F_{3,99} = 2.22$, $p = 0.90$), or their interaction ($F_{3,99}=1.23$, $p = 0.30$).

Immunoreactive Serum Oxytocin. As illustrated in Figure 3.2, dominant females had significantly higher immunoreactive serum levels of oxytocin compared with subordinate animals regardless of treatment condition ($F_{1,33}= 5.87$, $p = 0.02$) and this was unaffected by 5HTT genotype (Figure 3.2; $F_{1,33}$ 0.67, $p = 0.42$). In addition, E2 significantly ($F_{1, 33} = 4.15$, $p = 0.05$) increased overall immunoreactive serum oxytocin in all females (182 ± 14 vs. 209 ± 14) such that there was no status by E2 interaction on serum concentrations of immunoreactive oxytocin ($F_{1,33}$ 1.76, $p = 0.19$; data not shown). The administration of CRHA or its interaction with status or E2 treatment did not significantly affect immunoreactive serum oxytocin ($p > 0.05$; data not shown).

Anxiety-like behavior. Figure 3.3 shows E2 significantly attenuated anxiety-like behavior compared to placebo (6.07 ± 0.61 vs. 4.45 ± 0.49 per 30 min; $F_{1, 33} = 12.03$, $p < 0.01$). However, this anxiolytic effect of E2 was significantly modified by status and genotype ($F_{1, 33} = 5.22$, $p = 0.03$). Dominant females with an l/l 5HTT genotype had the lowest rates of anxiety-like behavior compared with other females ($p < 0.05$) and the decrease due to E2 replacement (13%) was not significant ($p > 0.05$). The higher rates of anxiety-like behavior were attenuated by E2 in dominant s-variant (25%) and subordinate l/l females (39%; $p < 0.05$) but not in subordinate s-variant females (9%; $p > 0.05$).

Finally, anxiety-like behaviors were not affected by treatment with the CRHA ($F_{1, 33}$ = 1.10, p = 0.30) or its interaction with E2, status, or genotype (p > 0.05).

Affiliative behavior. Overall, there were no main effects of status, 5HTT genotype, or a status by genotype interaction on affiliation directed toward others (p > 0.05). However, as shown in Figure 3.4A, treatment with E2 significantly increased affiliative behavior directed towards others compared to non-E2 treatment conditions (3.63 ± 0.36 vs. 2.76 ± 0.23 per 30 min; $F_{1, 33}$ = 4.91, p = 0.03). While the effect of E2 on increasing affiliation initiated by dominant, l/l females appeared to be less compared to other groups, there was no status by genotype by E2 treatment interaction on the amount of affiliative behavior initiated by females ($F_{1, 33}$ = 0.35, p = 0.56). Finally, affiliative behavior directed toward others was not significantly affected by CRHA treatment or its interaction with E2, status, or genotype (p > 0.05).

Corresponding to significantly higher rates of affiliation initiated by females during E2 treatments (Figure 3.4A), rates of affiliation received were also increased by E2 (2.80 ± 0.22 vs. 3.69 ± 0.30; Figure 4B; $F_{1, 33}$ = 7.24, p = 0.01). Dominant females were most often the targets of this behavior compared with subordinates regardless of treatment condition (3.80 ± 0.30 vs. 2.69 ± 0.28 per 30 min; Figure 4B; $F_{1, 33}$ = 7.07, p = 0.01). While it appeared the E2-induced increase in affiliation received was less in subordinates compared to dominant females, there was no status by E2 interaction ($F_{1, 33}$ = 2.23, p = 0.15). However, the effect of E2 varied significantly by status in the context of CRHA treatments (Figure 3.4B; $F_{1, 33}$ = 6.52, p = 0.02). In the absence of E2, CRHA decreased affiliation received by dominant females whereas rates were unchanged in subordinates. In contrast, treatment with CRHA reduced the rate of received affiliation

during E2 treatment in subordinate females whereas it increased rates during E2 for dominant females. Finally, 5HTT genotype did not modify the effects of status or E2 treatment whether females were targets of affiliation ($P > 0.05$).

Aggressive Behavior. As expected, dominant females initiated significantly more aggression than subordinate monkeys (3.44 ± 0.65 vs. 1.56 ± 1.56; Figure 3.5A; $F_{1, 33} = 4.78$, $p = 0.04$). However, s-variant females showed significantly higher rates of aggressive compared with l/l subjects (3.76 ± 0.59 vs. 1.21 ± 0.62; $F_{1, 33} = 8.94$, $p < 0.01$). There was no status by genotype interaction ($F_{1, 33} = 1.25$, $p = 0.27$). Importantly, as shown in Figure 5A rates of aggression directed toward others were significantly increased during treatment with E2 in all females (3.43 ± 0.65 vs. 1.57 ± 0.36; $F_{1, 33} = 9.35$, $p < 0.01$). In contrast, CRHA attenuated aggression (1.96 ± 0.31 vs. 3.04 ± 0.63; $F_{1, 33} = 5.00$, $p = 0.03$) regardless of E2, status or genotype ($p > 0.05$).

Not surprisingly, subordinate females were most often the target of aggression (Figure 3.5B; $F_{1, 33} = 7.67$, $p < 0.01$). Again, this status effect was modified by genotype ($F_{1, 33} = 4.63$, $p = 0.04$), as subordinate s-variant females received more aggression than subordinate l/l females (5.01 ± 1.14 vs. 2.10 ± 0.41). Corresponding to the higher rates of aggression initiated during E2 replacements, rates of aggression received were also higher during E2 ($F_{1, 33} = 5.00$, $p = 0.03$). The apparent decrease in aggression received by subordinates during CRHA treatment was not statistically significant ($F_{1, 33} = 1.85$, $p = 0.18$).

3.5 Discussion

Our current findings showed that mid-follicular phase levels of E2 increased behavioral activity, reflected in agonistic and affiliative behaviors, but provided support for the notion that exposure to stressors and upregulation of the stress axis attenuates the behavioral effects of E2 (Keen-Rhinehart et al., 2009; Pierce et al., 2008a; Uphouse et al., 2005) particularly in individuals that may be genetically more reactive to stressor exposure (Murphy and Lesch, 2008). While immunoreactive serum levels of oxytocin were lower in subordinate compared to dominant animals, regardless of 5HTT genotype, E2 increased oxytocin levels in all females. However, s-variant subordinate females showed highest rates of agonistic behavior and lowest rates of affiliation that occurred coincident with a reduced sensitivity to the anxiolytic actions of E2, suggesting that this 5HTT polymorphism synergizes with psychosocial stress exposure to affect the behavioral effects of E2. These findings are important for understanding the factors that modulate the behavioral response to E2 and for identifying mechanisms responsible for female emotional sociality and emotional well-being.

The present study extends previous observations of increased depressive- (Shively et al., 2006; Shively et al., 1997b) and anxiety-like behaviors (Wilson et al., 2008) related to social status in female macaques. While the lowest rates of anxiety-like behaviors were observed in dominant females with the l/l 5HTT genotype, dominant s-variant females had rates of anxiety indistinguishable from l/l subordinates. S-variant subordinates expressed anxiety behaviors at a higher rate than dominant l/l females but less than other groups. The higher rates of anxiety-like behaviors in subordinates may be an adaptive response to their unpredictable social environment (Huhman, 2006),

specifically potential threats of aggression (Troisi, 2002). Coupled with the higher rates of harassment, subordinates were less often recipients of affiliation and this too could contribute to the higher anxiety behaviors. However, this explanation does not hold for the dominant s-variant females, as their access to resources is unimpeded. Rather, their increased rates of anxiety may be a consequence of their propensity to aggress more frequently with cage mates, as reduced expression of 5HTT characteristic of the short allele of the 5HTT gene is associated with increased anxiety and impulsive behavior (Murphy and Lesch, 2008).

The well-established anxiolytic effects of E2 (Bernardi et al., 1989; Galea et al., 2001; Okada et al., 1997; Rocha et al., 2005; Walf et al., 2004) were significantly affected by social status and genotype. Rates of anxiety were decreased by E2 only in dominant s-variant and subordinate l/l females. It is possible that these behaviors could not be reduced further by E2 in the dominant l/l females, as their baseline level of anxiety-like behaviors were lowest compared to other groups. In contrast, the higher rates anxiety-like behaviors in s-variant subordinate females were unaffected by E2. This could be explained by a disruption of E2 efficacy in these animals, as limbic estrogen receptor levels are decreased in individuals with stress-induced affective disorders (Perlman et al., 2005; Perlman et al., 2004). However, this seems unlikely, as the s-variant subordinate females showed an increase in affiliative behavior during E2 replacement. It is possible that the neurochemical targets, notably 5HT, that mediate these anxiolytic effects of E2 are altered in some fashion in females exposed to the stress of subordination (Summers et al., 2003) and exacerbated by the s-variant polymorphism. Differences in response to the anxiolytic effects of E2 cannot be attributed to differences

in oxytocin, as there was no genotype difference in immunoreactive serum oxytocin in subordinate females. E2 dose-response studies on 5HT responsivity or central manipulation of the oxytocin system can better address these status - genotype differences in anxiety behavior.

Overall aggressive behavior was influenced independently by both status and 5HTT genotype. Specifically, dominant females were more aggressive towards subordinate cage mates. This is not surprising, as in such hierarchical social organizations, most individuals cannot avoid aggression received from higher-ranking group members and thus must emit submissive behaviors to terminate the aggression (Bernstein and Gordon, 1974). Furthermore, under the stable group situation of the present study, s-variant females were more aggressive than l/l subjects. Dysfunction of the 5HT system is linked to increased incidences of aggressive behavior, as 5HT usually acts to inhibit aggression (Summers et al., 2005) and limit impulsivity (Hollander and Rosen, 2000). Previous studies indicate 5HT tone is lower in individuals with an s-variant genotype (Hoffman et al., 2007; Manuck et al., 2004; Reist et al., 2001) and reduced central 5HT activity is associated with increased impulsivity and aggression (Higley and Linnoila, 1997; Hollander and Rosen, 2000; Manuck et al., 2003; Westergaard et al., 2003; Westergaard et al., 1999), as well as hostility in humans (Reist et al., 2003; Williams et al., 2003). Our data support the hypothesis that females with an s-variant 5HTT genotype are more aggressive in a stable social group situation.

Our observation that dominant animals received more affiliation than subordinate animals was associated with overall higher immunoreactive oxytocin levels present in dominant animals. Indeed, studies have shown that oxytocin facilitates affiliative

behavior (Campbell, 2008; Donaldson and Young, 2008) and promotes adaptive responses to challenging social situations (Lee et al., 2009). In addition, engaging in affiliative behavior enhances peripheral oxytocin levels (Carter et al., 2008; Paredes et al., 2006). E2 replacement increased both affiliative and aggressive behaviors in all females regardless of status and genotype. The increase in affiliative behaviors such as proximity and grooming by E2 is consistent with previous observations (Shively et al., 2007; Wallen and Tannenbaum, 1997). Furthermore, our data show that, in addition to increasing affiliative behavior, E2 also increased immunoreactive serum oxytocin in all females compared to the placebo condition. Our findings should be considered preliminary as E2-induced increases in OT have been reported only in the hypothalamus (Patisaul et al., 2003) and the link between changes in immunoreactive serum oxytocin and affiliative behavior is only correlational. These observations need to be confirmed with E2 dose-response changes in both peripheral and central oxytocin using an assay platform, such as mass spectrometry, that is independent of possible confounds associated with antibody affinity and specificity inherent in immunoassays.

Replacement of E2 to ovariectomized female rhesus monkeys also decreased morning cortisol levels, consistent with other data indicating E2 decreases basal or stress-induced activation of the LHPA axis (Patchev and Almeida, 1996; Saltzman et al., 2006; Young et al., 2001). However, other studies show that E2 increases activation of the LHPA axis (Viau and Meaney, 1991) by decreasing glucocorticoid negative feedback (Patchev and Almeida, 1996; Wilson et al., 2005); increasing adrenal sensitivity to ACTH (Figueiredo et al., 2007) and enhancing diurnal (Gudmundsson et al., 1999; Smith and Norman, 1987b) or morning cortisol secretion (Giussani et al., 2000; Stavisky et al.,

2003). While this discrepancy in the literature surrounding E2's ability to alter LHPA function might reflect differential access to social support (Barbosa and Mota, 2009; Doyle et al., 2008), a more parsimonious explanation is that a single morning cortisol sample was the only parameter of LHPA activity collected and not sufficient to adequately describe the effects of E2 on LHPA function. While the lack of a social status difference in morning cortisol levels during the placebo treatment could be considered a limitation of the current study, these data are consistent with previous studies suggesting that using a single measure of morning cortisol is not sufficient measure of LHPA activity in subordinate monkeys (Michopoulos et al., 2009a; Michopoulos et al., 2010). Assessing LHPA negative feedback by dexamethasone administration is necessary to show hypercortisolemia in subordinate females (Kaplan et al., 1984; Sapolsky, 2005; Shively, 1998b; Wilson et al., 2008).

Administration of a 10 mg/kg dose of the CRH type 1 receptor antagonist CP154,526, an analog of the widely used antalarmin, for five consecutive days paradoxically increased serum cortisol levels in all females even in the presence of E2. Previous uses of CP154,526 on glucocorticoid levels and anxiety behaviors are inconsistent, in both rodents (Arborelius et al., 2000; Bornstein et al., 1998) and non-human primates (Ayala et al., 2004; French et al., 2007; Habib et al., 2000). While the effects of the CRHA on behavior were associated with increased cortisol levels, we cannot rule out the possibility that the CRHA is having a more direct effect on modulating social behaviors via a central mechanism, and thus this data should be considered preliminary. Further studies are necessary to determine the mechanism by

which increased cortisol levels mediate aggression and affiliation in female rhesus monkeys.

In summary, the data reported here add support to the long-standing notion that E2 has potent effects on female socio-emotional behavior and is consistent with the hypothesis that E2 induces generalized behavioral arousal, allowing the female to adapt to and cope with environmental challenges (Ribeiro et al., 2009). However, the data extend these findings by showing how social subordination and 5HTT genotype may modify these effects. The attenuated anxiolytic response to E2 in subordinate s-variant females supports data showing exposure to stressor may disrupt E2 regulation of behavior sensitivity to E2 and further implicates the s-variant of the 5HTT polymorphism as a predisposing factor in increased individual vulnerability to adverse consequence due to psychosocial stress exposure. Furthermore, the present data suggest that social status differences in immunoreactive serum oxytocin, as a surrogate measure of central concentrations, may be one of several neurochemical factors that mediate the expression of these social behaviors. We must also emphasize that, in social living animals, socio-emotional behaviors do not occur in isolation but rather reflect a female's response to her social environment and biological condition. Thus, an evaluation of the hormonal regulation of these behaviors must take that of a multi-variable approach into consideration. Dose – response studies with E2 are needed to better define the parameters and neurochemical basis of reduced sensitivity to E2 in this model of psychosocial stress.

Table 3.1. Mean ± SEM serum concentrations of morning cortisol (μg/dl) during each of the four treatment conditions in dominant females and subordinate females with an l/l or s-variant 5HTT genotype. Estradiol administration significantly attenuated serum cortisol ($p < 0.001$), indicated by different numbered superscripts. However, CRHA (CRH receptor analogue) administration significantly increased serum cortisol ($p = 0.001$), indicated by a different lettered superscript. Serum cortisol did not differ significantly by status, genotype, or their interactions with treatments. [*From (Michopoulos et al., 2011b)*]

Group	**Placebo**[1, A]	**CRHA**[1, B]	**Estradiol**[2, A]	**CRHA + Estradiol**[2, B]
Dom, l/l	27.9 ± 1.8	31.9 ± 2.4	23.0 ± 2.0	30.2 ± 2.3
Dom, s-variant	29.5 ± 2.6	34.6 ± 2.9	27.4 ± 1.9	30.6 ± 2.9
Subordinate, l/l	27.4 ± 1.7	32.1 ± 2.3	24.7 ± 1.8	28.4 ± 2.8
Subordinate, s-variant	28.6 ± 1.4	33.0 ± 1.9	26.3 ± 1.6	32.5 ± 2.4

Figure 3.1. Mean ± SEM rates of agonistic behavior collapsed across treatment conditions and genotype. Animals categorized as dominant (ranked 1 and 2) received less aggressive behavior (closed circle) than those categorized as subordinate (ranked 3-5) while subordinate animals emitted more submissive behaviors (open square) than dominant animals. [*From (Michopoulos et al., 2011b)*]

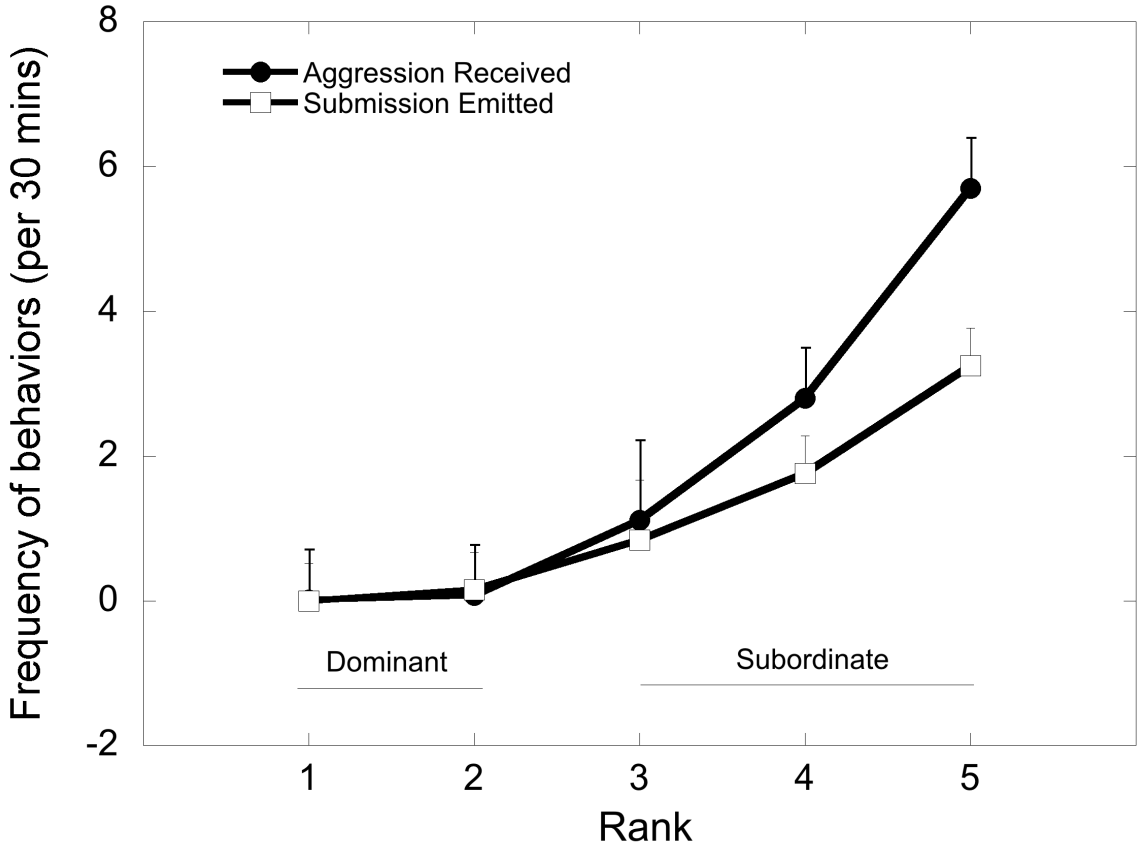


Figure 3.2. Mean ± SEM serum concentrations of oxytocin in dominant and subordinate females with either an l/l (open bar) or s-variant (closed bar) 5HTT genotype. Values presented are overall oxytocin levels, collapsed across E2 (estradiol) and CRHA (corticotropin releasing-hormone receptor analogue) treatment conditions. [*From (Michopoulos et al., 2011b)*]

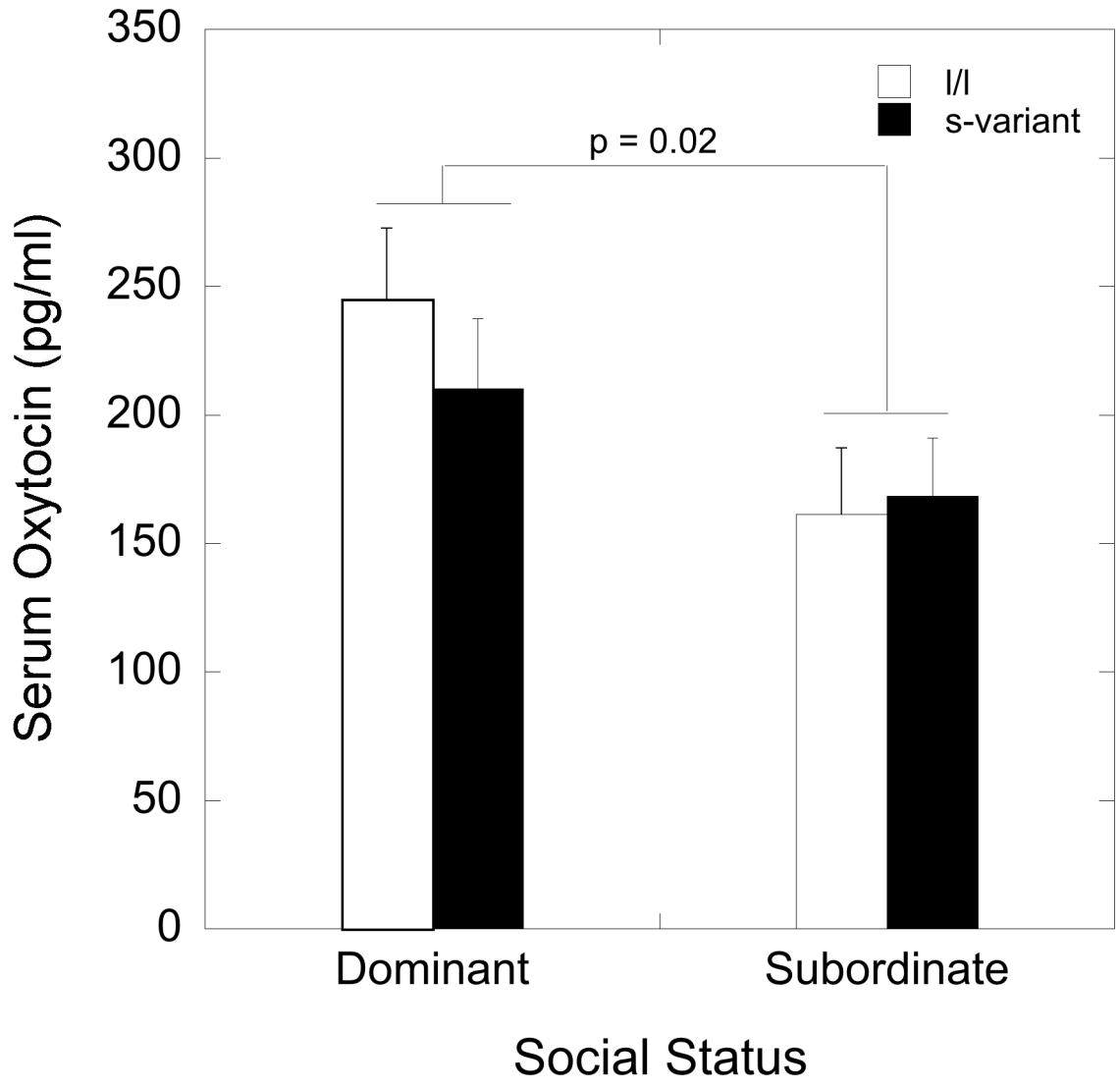


Figure 3.3. Mean ± SEM rates of anxiety-like behavior in dominant females and subordinate females with either an l/l or s-variant 5HTT genotype during the placebo (open bar), estradiol (hatched bar), corticotropin releasing-hormone receptor analogue (CRHA; grey bar), and combined estradiol and CRHA (closed bar) treatments. Different letters among the four study groups reflect significantly different rates of anxiety-like behavior collapsed across treatment conditions. The asterisk indicates estradiol replacement significantly attenuated anxiety-like behaviors in those study groups. [*From (Michopoulos et al., 2011b)*]

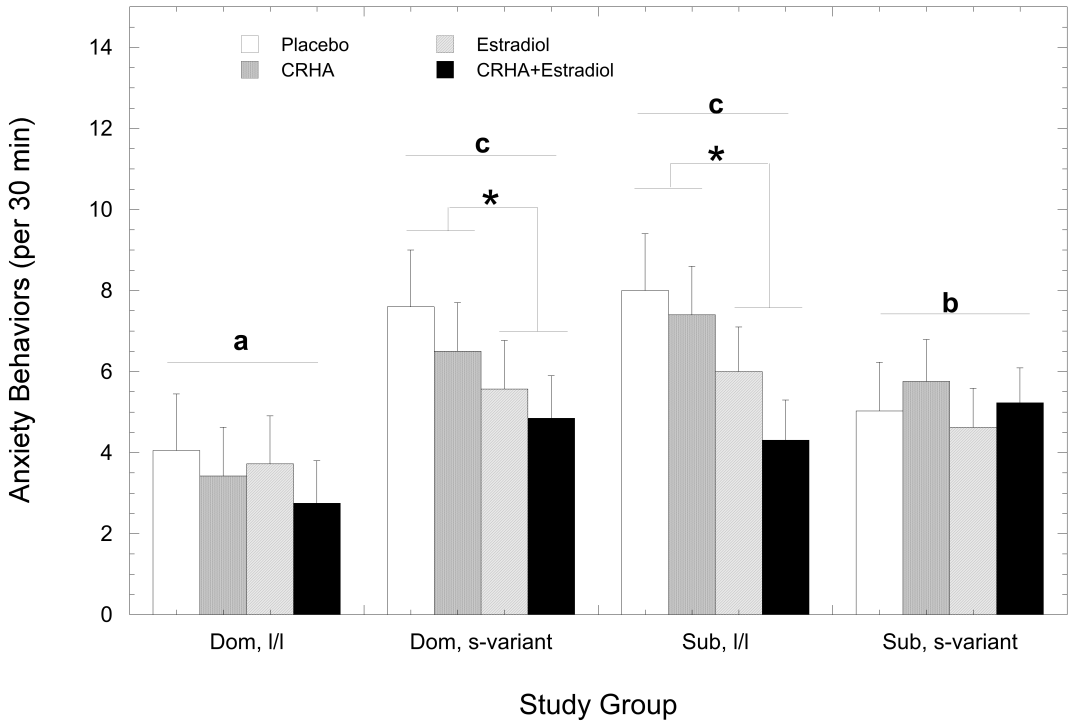


Figure 3.4. Mean ± SEM rates for affiliative behavior (A) directed towards others and (B) received from others during the placebo (open bar), estradiol (hatched bar), corticotropin releasing-hormone receptor analogue (CRHA; grey bar), and combined estradiol and CRHA (closed bar) treatments in dominant females and subordinate females with either an l/l or s-variant 5HTT genotype. Asterisks indicate the significant elevation in behavior induced by estradiol replacement. [*From (Michopoulos et al., 2011b)*]

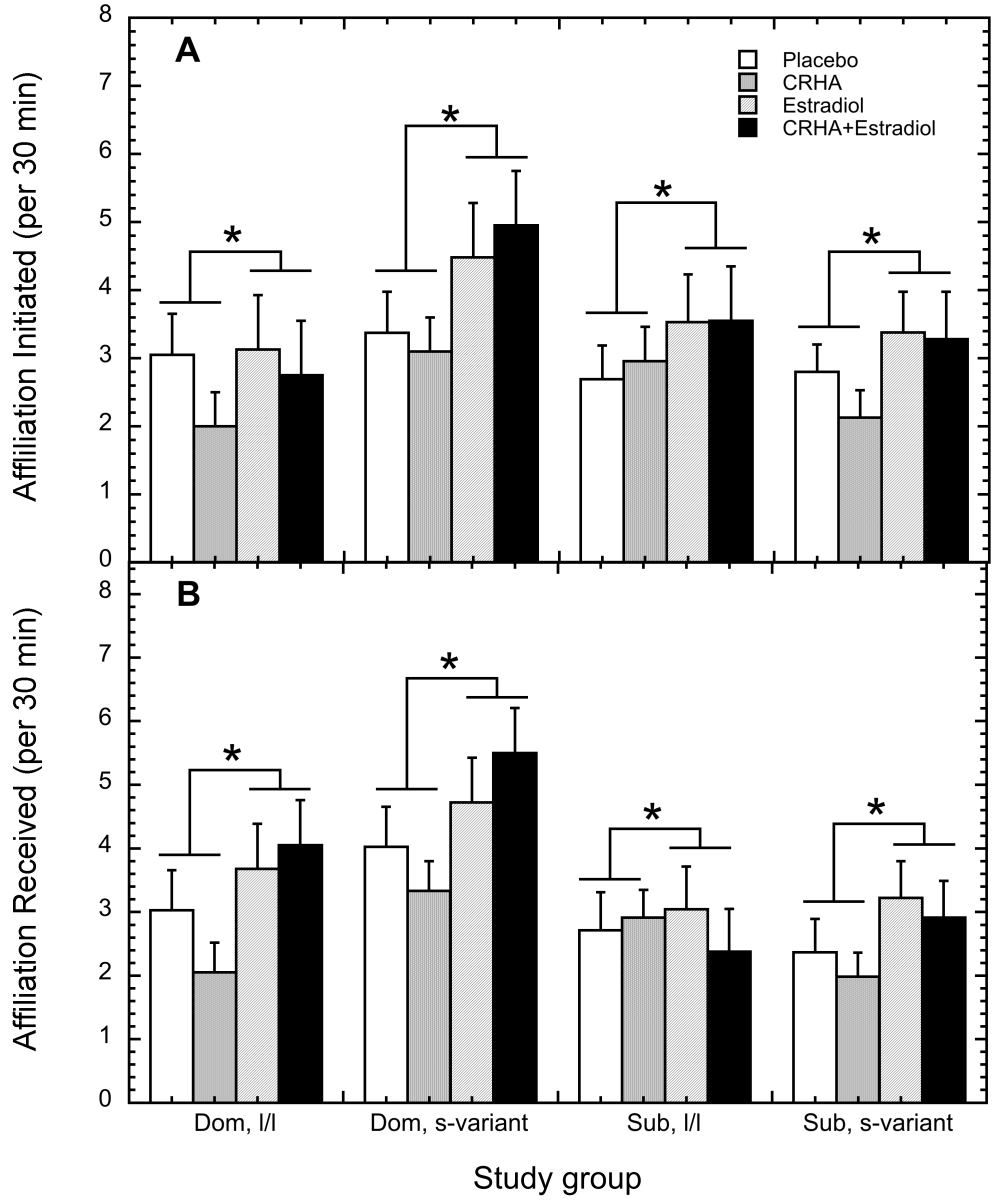
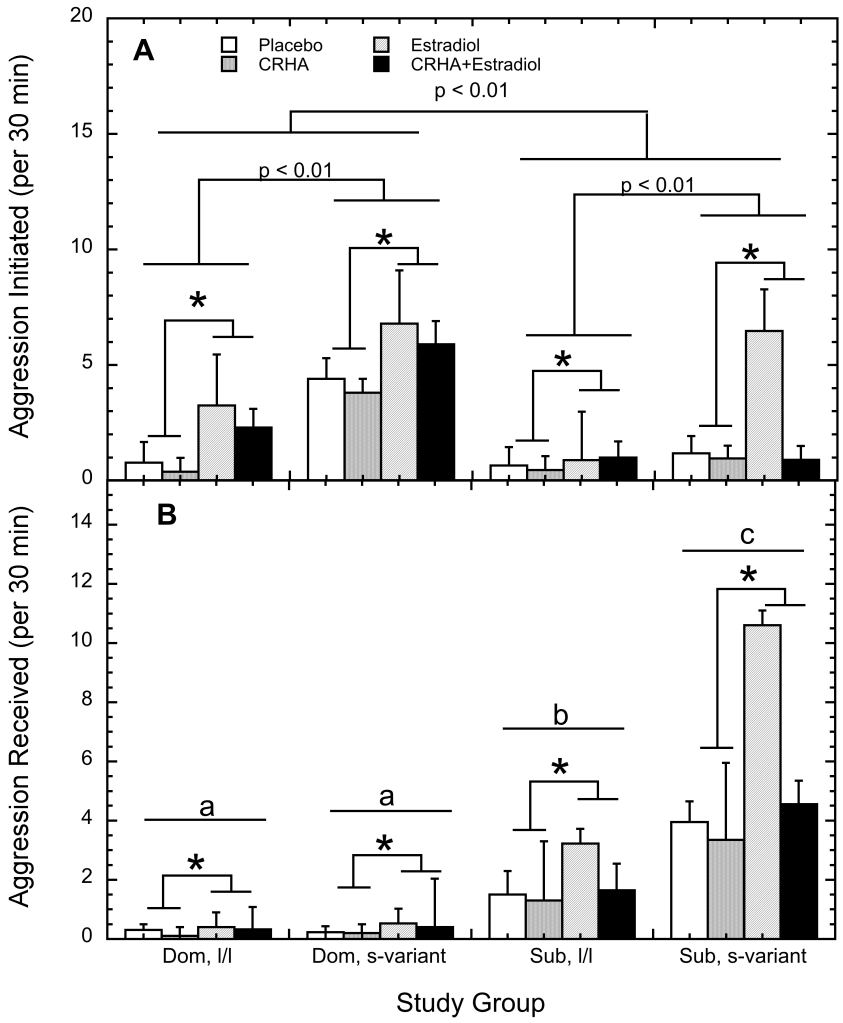


Figure 3.5. Mean ± SEM rates for aggressive behavior (A) initiated and (B) received during the placebo (open bar), estradiol (hatched bar), corticotropin releasing-hormone receptor analogue (CRHA; grey bar), and combined estradiol and CRHA (closed bar) conditions in dominant females and subordinate females with either an l/l or s-variant 5HTT genotype. Asterisks indicate the significant increase in behavior induced by estradiol replacement. Different letters in panel B indicate groups different significantly from one another in aggression received regardless of treatment phase ($p < 0.05$). [*From (Michopoulos et al., 2011b)*]



CHAPTER FOUR:

SOCIAL SUBORDINATION INTERACTS WITH DIET HISTORY TO PROMOTE EMOTIONAL FEEDING IN FEMALE RHESUS MONKEYS

[All text and figures from (Michopoulos et al., 2012b)]

4.1 Abstract

Stress-induced eating disorders cause significant health problems and are often co-morbid with mood disorders. Emotional feeding, particularly in women, may be important for the development of obesity and failed attempts to lose weight. However, prospective studies assessing the effect of chronic psychosocial stress on appetite in different dietary environments in females are lacking. The present study tested the hypothesis that chronic psychosocial stress would increase consumption of high caloric diet and this emotional feeding would persist even when a healthier diet was available. Socially housed female rhesus monkeys were studied to address whether subordination increases caloric intake when a high fat and sugar diet (HFSD) was available concurrently with a low fat, high fiber diet (LCD). Cortisol responsivity and food intake was quantified during this choice phase and when only the LCD was available. The order of diet condition was counterbalanced to assess whether a history of HFSD would affect a]ppetite. All females preferred the HFSD but subordinates consumed significantly more calories from both diets combined during the choice phase. The increased calorie intake was maintained in subordinate monkeys even after withdrawal of the HFSD. Subordinate females demonstrated reduced glucocorticoid negative feedback, with post dexamethasone serum cortisol levels significantly predicting intake of the HFSD but not the LCD during the choice condition. The cortisol response to an acute stressor significantly predicted subsequent intake of a HFSD in all females. Thus, continual exposure to the psychosocial stress of subordination in female monkeys results in excess caloric intake of foods that mimic a western dietary environment. In addition, this social

stressor interacts with a history of HFSD intake to promote increased feeding even in a healthy dietary environment.

4.2 Introduction

In 2009, nearly 73 million adults in the US were obese, representing 28% of the population and an increase of 7% over 2001 rates (Organization, 2010). Additionally, 34% of adults in the US are overweight (Flegal, 2005). Because the health (Hill, 2006) and economic burden (Withrow and Alter, 2011) imposed by obesity is enormous, effective programs to prevent or alleviate obesity are a high priority. While gene variants regulating satiety or metabolism are known to influence appetite and body weight control (Hinney et al., 2010), environmental triggers are likely key determinants of this phenotype (Font et al., 2010). Indeed, emotional feeding resulting from the chronic exposure to psychosocial stressors is a probable contributing factor for excess food intake (Bjorntorp, 2001; Dallman et al., 2005; Rosmond, 2004; Scott et al., 2008). Importantly, attempts to lose weight often fail (Kassirer and Angell, 1998), as eating behaviors become disinhibited and people overeat in response to emotional states (Hays and Roberts, 2008).

Emotional feeding is coincident with both periods of acute and chronic exposure to psychosocial stressors (Adam and Epel, 2007). Psychopathologies with etiologies related to chronic exposure to psychosocial stressors, such as depression and anxiety disorders, are highly comorbid with obesity (Simon and Arterburn, 2009; Werrij et al., 2006). It is uncertain which factors increase an individual's vulnerability to augment caloric intake under stressful circumstances. While intake of highly palatable foods is rewarding via activation of the dopaminergic reward system (Bassareo and Di Chiara, 1999; Blackburn et al., 1986; Johnson and Kenny, 2010; Small et al., 2003), it remains

unclear how consumption of a calorically dense diet or one that is high fat and sugar diet (HFSD) alters physiological responses to both chronic and acute stressors.

There is a great deal of uncertainty surrounding the effects of HFSD consumption on the activity of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis in the literature. This could be due to a number of factors including the specific animal model employed and whether a dietary choice was available as a part of the stress paradigm (Adam and Epel, 2007; Warne, 2009). Another important consideration for the study of stress-induced emotional feeding that is often neglected in animal models is gender. Indeed, emotional eating (Zellner et al., 2006; Zellner et al., 2007) and an obese phenotype (Barry et al., 2008; Jones and Carney, 2006; Weissman and Olfson, 1995; Wurtman, 1993; Wurtman and Wurtman, 1995) occur significantly more often in women. The discrepancies in the current animal models available, and the alarming rate at which obesity epidemic is rising, necessitates that an appropriate animal model of stress-induced eating is developed so that the mechanisms responsible for stress-induced caloric intake can be elucidated.

Social subordination in female rhesus monkeys (Macaca Mulatta) is a well-characterized ethologically relevant, translational animal model used to study the adverse health effects of chronic psychosocial stress exposure in women (Adams et al., 1985; Cohen, 1999; Gust et al., 1991; Jarrell et al., 2008; Kaplan et al., 1996; Michopoulos et al., 2009a; Morgan et al., 2002; Paiardini et al., 2009; Sapolsky, 2005; Shively, 1998b; Wilson et al., 2008). We tested the hypothesis that socially subordinate females would increase overall caloric intake in a dietary environment mimicking a typical western situation wherein both low fat, high fiber diet and a HFSD were made available. In

addition, we tested the hypothesis that a history of HFSD consumption would interact psychosocial stress promote excess calorie consumption even in a healthier dietary environment.

4.3 Material and methods

Animals. Previously ovariectomized adult female rhesus monkeys (n = 39) living in indoor-outdoor enclosures at the Yerkes National Primate Research Center (YNPRC) Field Station (in groups of 4 and 5 females and 1 male) were used as subjects. No hormone replacement was used during the study. All animals had access to experimental diets ad libitum via previously validated automated feeders that allow for constitutive quantification of individual caloric intake (Arce et al., 2010; Wilson et al., 2008). Briefly, activation of a radio-frequency (RF) antenna via a RF identification chip within each animal's wrists signaled a computer to dispense a single pellet of food via a pellet. Each group of animals had access to two different feeder systems and a computer recorded each feeding event in a log. Dominant females rarely (<1%) take the pellet from subordinate animals and pellets are never discarded (Wilson et al., 2008). The Emory University Institutional Animal Care and Use Committee approved all procedures in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for Care and Use of Laboratory Animals."

Social stability in macaque groups, regardless of size, is maintained by a dominance hierarchy (Bernstein and Mason, 1970). Lower-ranking individuals in a social group receive a greater frequency of aggression from higher-ranking group mates and emit higher levels of submissive behaviors towards these more dominant individuals. A direct consequence of low social status in female rhesus monkeys is reduced control

over both social and physical environments (Sapolsky, 2005) that result in disruption of LHPA function including diminished glucocorticoid negative feedback (Jarrell et al., 2008; Shively, 1998b; Wilson et al., 2008). Therefore, social subordination in female rhesus monkeys is a well-characterized model with which to study the negative effects of chronic psychosocial stress exposure on behavior and physiology, including reproductive dysfunction (Adams et al., 1985; Michopoulos et al., 2009a), immune compromise (Gust et al., 1991; Paiardini et al., 2009), addictive behavior (Morgan et al., 2002), and cardiovascular disease (Kaplan et al., 1996). In the current study, the outcome of dyadic interactions between females obtained from four 30-minute observations throughout each study phase using an established ethogram (Jarrell et al., 2008), was used to establish group dominance ranks. As previously described (Kaplan et al., 1984), females ranked one and two were classified as dominant (n=15) and females ranked 3-5 were considered as subordinate (n=24). Social groups had been formed and dominance ranks stable for 108 months prior to the initiation of this study.

Experimental Design. Females were studied for two two-week diet condition phases separated by a three-week washout period. Each phase consisted of either a dietary choice between a low calorie monkey chow (LCD; 3.45 kcal/g, Purina 5038) and a high fat and sugar diet (HFSD; 3.73 kcal/g Purina Typical American Diet #5038) or access to only the LCD (no choice condition). The order of diet condition presentation was counterbalanced so that half the females received the choice condition first and the other half the no choice condition first. The caloric composition of the LCD was 12% fat, 18% protein, and 4.14% sugar carbohydrate and 65.9% fiber carbohydrate. The

calories of the HFSD were distributed as 36% fat, 18% protein, 16.4% sugar carbohydrate and 29.6% fiber-starch carbohydrate.

Body weights and non-fasted blood samples for the analysis of serum leptin, insulin, and glucose were taken at the beginning and end of each phase of the study at 0800 hr to assess changes due to diet availability. To assess the effects of social status on glucocorticoid negative feedback, a dexamethasone suppression test (DST) was administered the week prior to the start of the study. Serum samples were collected at 0800, 1100, and 1730 hr. Immediately following the sample at 1730 hr, females received the dexamethasone injection (0.25 mg/kg, IM). Samples were collected the following morning at 0800 and 1100 hr for cortisol analysis.

To assess the effects of diet availability and intake on cortisol response to an acute stressor, all females were exposed to a social separation stressor (SSS) paradigm on the second week of each diet phase. Isolation of a female rhesus monkey from her social group and confinement for 30 min results in a significant increase in serum cortisol levels (Collura et al., 2009). Blood samples for cortisol analysis were obtained at time 0 (1300) and 40 min later, after which animals were returned to their home caging.

Statistical analyses. Data were summarized as mean ± standard error of the mean (SEM). The main and interaction effects of status (dominant vs. subordinate), order of diet presentation (choice first vs. no choice first), diet condition (choice vs. no choice), weeks, days, and time of day on caloric intake, metabolic measures, and cortisol response to an acute stressor were evaluated with analysis of variance for repeated measures (RM-ANOVA) using SPSS 19 (IBM). Preliminary analysis showed that 5HTT genotype did not influence LHPA activity as assessed in this study and thus 5HTT was consequently

dropped from the analyses. The feeding data were transformed with a log10 to correct for the lack of homogeneity of variance. The change in cortisol levels due to the SSS was analyzed by RM-ANOVA to assess the effects of diet availability and history on LHPA response to an acute stressor. Finally, Pearson product moment correlations were used to assess the association between selected variables and caloric intake. Test results with a $p \leq 0.05$ were considered significant and post-hoc analysis conducted when necessary.

4.4 Results

Social Status based on agonistic behavior and DST. As shown in Figure 4.1A, rates of aggression received and submissive behavior emitted for monkeys at each social dominance rank position. Social rank significantly affected the amount of aggression received ($F_{4, 29} = 8.15$, $p<0.001$) and submission emitted by females ($F_{4, 29} = 5.13$, $p=0.003$). Categorizing females ranked 1 and 2 as dominant and those ranked 3 through 5 as subordinate results in a significant main effect of status on submissive behaviors emitted ($F_{1, 35} = 13.6$, $p=0.001$) and increased levels of aggression received ($F_{1, 35} = 10.0$, $p=0.003$).

Glucocorticoid negative feedback, assessed by suppression of cortisol following dexamethasone administration, was affected significantly by a time – status interaction ($F_{1, 35} = 4.41$, $p=0.049$). Comparing cortisol concentrations following dexamethasone to those obtained at baseline the previous day showed that the change in cortisol at 0800 due to dexamethasone suppression was significantly greater in dominant females than in subordinate females ($p=0.034$; Figure 4.1B). At 1100, dominant animals had similar levels of cortisol as subordinate females ($p>0.05$).

Effects of diet availability on calorie intake. Irrespective of the initial diet condition (choice or no-choice), total caloric intake was significantly affected by the interaction of social status and diet condition ($F_{1, 35} = 4.67$, p=0.034; Figure 4.2). Dominant females ate a similar number of total calories during the no choice and choice diet conditions ($F_{1, 28} = 2.28$, p=0.142; Figure 4.2). Subordinate females, however, consumed significantly more calories during the choice condition compared to the no choice diet condition ($F_{1, 35} = 4.19$, p=0.048; Figure 4.2). During the choice condition, subordinate females ate more calories from the LCD than did dominant females (p=0.05; Figure 4.2). Both dominant and subordinate females preferred the HFSD to the LCD during this choice condition ($F_{1, 37} = 7.10$, p=0.011; Figure 4.2) and, while it appeared subordinate females consumed more calories from the HFSD than did dominant animals, the difference was not significant (p=0.121). Nonetheless, during the choice phase, total caloric intake was greater in subordinates compared with dominant monkeys (p=0.01). This preference for the HFSD over the LCD was most evident during the daytime (26.0 ± 4.35 vs. 14.3 ± 1.57 kcal/hr) than during nighttime feeding (10.1 ± 2.44 vs. 4.8 ± 1.42 kcal/hr). Finally, the intake of the HFSD during the choice condition was significantly predicted from both baseline ($r_{37} = 0.33$, p = 0.04) and post dexamethasone serum concentrations of cortisol ($r_{37} = 0.39$, p = 0.02). Furthermore, these parameters of cortisol secretion also significantly predicted total caloric intake during the choice condition (baseline: $r_{37} = 0.33$, p = 0.04; post-dexamethasone: $r_{37} = 0.44$, p = 0.01) but not LCD during the choice condition (baseline: $r_{37} = 0.05$, p = 0.77; post-dexamethasone: $r_{37} = 0.15$, p = 0.37). Similarly, neither parameter of cortisol secretion was associated

with intake of LCD during the no choice condition (baseline: $r_{37} = 0.27$, $p = 0.09$; post-dexamethasone: $r_{37} = 0.26$, $p = 0.12$).

Importantly, the order of diet phase, whether animals received the no choice or choice condition first, had a significant effect on caloric intake ($F_{1, 35} = 7.12$, $p=0.011$), and this effect was modified by social status ($F_{1, 35} = 4.07$, $p=0.05$), Table 4.1, Figure 4.3). The total number of calories consumed in the two diet conditions did not vary significantly by order of diet presentation in dominant females, ($F_{1, 13} = 0.002$, $p=0.966$). Similarly, when the no choice preceded the choice phase, subordinate females consumed a similar number of calories during this LCD-only condition compared with that of the dominant animals ($p=0.479$). In contrast, if the choice condition came first, subordinate females consumed significantly more calories during the subsequent no choice phase than did dominant females (749 ± 87 vs. 480 ± 112 kcal/day, $p=0.030$; Figure 4.3) and significantly more than subordinate counterparts who had no history of HFSD intake (749 ± 87 vs. 356 ± 83 kcal/day) ($p=0.006$; Figure 4.3), suggesting exposure to a HFSD diet changed appetite regulation for a healthier LCD in subordinates but not dominant females.

Effects of diet availability on cortisol response to an acute stressor. The increase in cortisol following the social separation was significantly greater during the diet choice phase compared to the no choice condition ($F_{1, 35} = 20.5$, $p<0.001$; Figure 4.4). This effect of diet condition on cortisol responsivity was not affected by social status ($F_{1, 35} = 0.339$, $p=0.564$) or diet order (data not shown; $F_{1, 35} = 0.013$, $p=0.909$). However, during the choice dietary condition, the change in serum cortisol following the social separation significantly predicted caloric intake of the HFSD ($r_{35} = 0.44$, $p = 0.007$) but

not the LCD ($r_{35} = 0.01$, p=0.976; Figure 4.5) in all females. In contrast, the change in serum cortisol following the social separation did not predict subsequent intake of the LCD during the no choice phase ($r_{35} = 0.06$, p = 0.70).

Effects of diet availability on body weight and serum hormones. Prior to the start of the study and while females were maintained on the LCD, dominant females (9.26 ± 0.40 kg) weighed significantly more than subordinates (7.60 ± 0.29 kg; $F_{1, 35} = 11.1$, p=0.002), a phenotype consistent with earlier reports of socially housed female rhesus monkeys (Jarrell et al., 2008). As illustrated in Table 4.2, the change in body weight was significantly different during the no choice (-0.18 ± 0.04 kg) compared with the choice diet phases (-0.01 ± 0.04; $F_{1, 35} = 11.1$, p=0.002). The diet phase dependent change in body weight was not affected by social status ($F_{1, 35} = 1.32$, p=0.259) or diet order ($F_{1, 35} = 2.66$, p=0.112).

At the start of the study and while females had been maintained on the LCD, dominant females (15.61 ± 2.01 ng/ml) had significantly higher serum levels of leptin more than subordinates (8.53 ± 1.51 ng/ml; $t_{1, 37} = 2.81$, p = 0.01), a phenotype again consistent with earlier reports of socially housed female rhesus monkeys (Jarrell et al., 2008). During the course of the study, social status and diet condition interacted to significantly affect the change in serum leptin over the two weeks of the choice vs. the no choice phase ($F_{1, 35} = 8.04$, p=0.008; Table 4.2), as serum leptin increased significantly during the choice condition in subordinates (p=0.005), but not dominant females (p=0.196; Table 4.2) regardless of order of diet condition.

At the start of the study and while females had been maintained on the LCD, serum insulin was higher in dominant compared to subordinates but the difference was

not significant (103.2 ± 23.0 vs 73.8 ± 14.5 IU/ml, $t_{37} = 1.25$, $p = 0.22$). Serum concentrations of insulin and glucose at the end of the LCD-only or choice diet condition, regardless of diet order, did not vary significantly by status ($F_{1, 35} = 1.13$, $p=0.296$; $F_{1, 35} = 2.74$, $p=0.107$, respectively; Table 4.2). However, the change in insulin levels was significantly affected by a diet condition – order of diet availability interaction ($F_{1, 35} = 6.67$, $p=0.014$; Table 4.2). When the LCD-only condition was first, serum insulin increased significantly during the choice condition relative to the LCD-only condition ($p=0.005$). However, when the choice condition was first, the increase in serum insulin during the choice phase was similar to the subsequent LCD only phase ($p=0.906$; Table 2). Although the increase in serum insulin was greater in subordinates during the choice condition, the difference was not significant ($p > 0.05$).

A similar pattern prior to any diet intervention was observed for serum glucose, with non-significantly higher levels in in dominant females (105.3 ± 4.9 vs. 95.47 ± 2.6 mg/dl, $t_{37} = 1.77$, $p = 0.09$). Serum glucose levels were higher in all females at the end of the choice compared to the no choice condition (98.0 ± 2.37 versus 91.4 ± 2.33; $F_{1, 35} = 4.60$, $p=0.039$). A diet condition – order of diet presentation effect was also seen in glucose levels ($F_{1, 35} = 7.63$, $p=0.009$). Glucose levels were not different between the two diet conditions when the LCD-only condition was first ($p=0.262$). However, when the choice condition was first, decreased during the choice condition but increased during the no choice condition ($p=0.001$; Table 4.2).

4.5 Discussion

Results here, summarized in Table 4.3, suggest that the chronic psychosocial stress experienced by subordinate females increases calorie intake only when these females are exposed to a dietary environment similar to human beings, where both a LCD and HFSD are available. These data are in agreement with a number of studies in male rodents (Dallman et al., 2007; Foster et al., 2006; Warne, 2009) and people (Epel et al., 2004; Torres and Nowson, 2007; Wallis and Hetherington, 2009) that show that stressor exposure increases intake of calorically dense diets. However, our data extend these findings to show that a history of HFSD consumption sustains excess calorie intake of the healthier LCD following removal of the HFSD choice in females experiencing the chronic stress of social subordination but not dominant females whose LHPA axis is more tightly regulated. Indeed, dominant females consume a similar number of calories regardless of diet availability or history. This pattern of emotional feeding in subordinate females is associated with a dysregulation of feedback inhibition of the LHPA axis, a phenotype similar to many stress-induced disorders in people (Chrousos, 2009).

The significantly greater food intake by subordinates in a calorically rich dietary environment is consistent with other data showing exposure to chronic stress increases food intake (Arce et al., 2010; Tamashiro et al., 2006; Warne, 2009). However, we did not observe a significant increase in body weight in subordinates despite the substantial increase in caloric intake. It is possible that the increased energy intake was obviated by increased activity and energy expenditure in subordinates (Solomon et al., 2011). However, it could also be that that the duration of access to the HFSD was too short, as we have previously shown that three-week access significantly increases body weight in

subordinates (Arce et al., 2010). Novel, however, is the finding that the consuming a HFSD also increases consumption of low fat, low sugar foods but only in subordinate females. This was not the case prior to the availability of the HFSD because groups that started initially with the no-choice LCD condition did not show any difference in caloric intake between subordinate and dominant females (347 ± 112 vs. 356 ± 87 kcal/day). These data imply that in an environment where a choice of diets with different palatable qualities is available, females exposed to continual psychosocial stressors increase total overall caloric intake, not just excess calories from highly palatable food.

In addition, exposure to a high fat, high sugar diet interacted with social subordination to produce changes in subsequent caloric intake after the highly palatable diet had been removed. Dominant animals consumed a similar number of calories during both diet conditions regardless of order of diet presentation, indicating diet history had no lasting effect on food intake in these animals. However, when the diet choice condition preceded the no choice phase, subordinates ingested more calories than did dominant females during the subsequent no-choice diet condition and significantly more calories than subordinates who had the no choice condition first and no previous history of HFSD consumption (Table 4.2, Figure 4.3). These data support our previous preliminary observations (Arce et al., 2010) and suggest that exposure to psychosocial stress interacts with diet history to promote additional caloric intake even in a healthy dietary environment. The lasting effect of stress and experience of comfort food ingestion may account for the high probability of failure observed in human beings when attempting to lose weight by adopting a healthier dietary environment after years of eating calorically dense foods (Johnson and Kenny, 2010; Kassirer and Angell, 1998).

Presentation of the diet choice first resulted in an increase in both insulin and glucose levels in all females that remained high during the following no choice diet condition. Because total calorie consumption was not different in any of the conditions for dominant females, these data raise the possibility that the lasting effect of HFSD exposure on caloric intake in subordinate females may be due to a synergistic stress- and HFSD-induced loss of sensitivity to satiety signals, like insulin and leptin that are critical for maintaining homeostatic energy balance (Lustig, 2008). In rats, the combination of chronic stress and a high-fat diet exacerbates insulin resistance compared to the effect of either a high fat diet or chronic stress alone (Fu et al., 2009). As evidence of this, serum leptin levels were increased in subordinate but not dominant females during the choice diet condition, yet subordinates maintained high caloric intake in the following no-choice phase. In monkeys (Arce et al., 2010) and in rats (Mietus-Snyder and Lustig, 2008), increased levels of glucocorticoids produce overeating and weight gain despite increased leptin levels, suggesting that glucocorticoids may lead to leptin insensitivity. Although serum glucose was not elevated during the choice phase in subordinates, continued access to this rich dietary environment may have significantly reduced insulin sensitivity, increased serum glucose and worsened leptin sensitivity (Lustig, 2008).

Based on studies in rodents, a possible explanation for increased consumption of calories by subordinate female monkeys when a HFSD is available is that intake of a palatable diet acts to reduce LHPA reactivity, most typically shown by an attenuation of the LHPA response to an acute stressor in previously stressed animals given access to a dietary environment that includes a choice of macronutrients (Dallman et al., 2005; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2010). These observations in

male rats are supported by data showing obese women who report a high degree of emotional feeding have a reduced cortisol response to an acute stressor (Tomiyama et al., 2011). Furthermore, the data from rodents argue that availability of a choice between a LCD and a sugar or fat diet is important to show this effect of stress hormone attenuation by diet (Warne, 2009). We chose to present females with a choice between a LCD and HFSD rather than just a HFSD condition as we felt it more closely mimicked the dietary environment available to people. Nonetheless, we observed that the increase in serum cortisol following the social separation stressor was significantly higher during the choice diet condition compared to the no choice diet condition regardless of social status or order of the diet regimen. This result is consistent with our previous data showing that intake of a calorically dense diet augments the cortisol response to an acute stressor in both dominant and subordinate animals (Arce et al., 2010). Our data are consistent with those showing intake of calorically dense foods increases LHPA activity in rodents (Kamara et al., 1998; Tannenbaum et al., 1997) and in humans (Pasquali et al., 2002). The most parsimonious explanation for this is that when more energy resources are available, due to increased caloric intake, more cortisol is released to mobilize these increased energy resources. Furthermore, data showing that glucocorticoids are key signals changing food salience and increasing caloric intake (Warne, 2009) are consistent with the notion that emotional feeding is sustained by an activation and not a diminution of the LHPA axis. Indeed, data from the present study show that post dexamethasone and post acute stressor serum levels of cortisol significantly predict intake of a HFSD but not a LCD. These data suggest that acute stress superimposed on chronic stress may promote continued intake of high caloric diets. Nonetheless, additional prospective

studies are needed to better understand the neuroadaptations that sustain emotional feeding in the face of chronic stress.

An important consideration in understanding the factors the initiate and sustain emotional feeding are possible sex differences. Human data indicate that women (Zellner et al., 2006) and not men (Zellner et al., 2007) most often show emotional feeding in response to socio-emotional stressors and are twice as likely as men to suffer from eating and affective disorders (Barry et al., 2008; Weissman and Olfson, 1995; Wurtman and Wurtman, 1995). One could argue that the well-established sex difference in stress responsivity (Dalla et al., 2005; Handa et al., 1994a; Kaplan et al., 1996; Kirschbaum et al., 1992; Young, 1998) may translate to differences in stress-induced ingestion of calorically dense diets. Nonetheless, data from male rodents clearly show males that experience restraint (Dallman et al., 2007; Warne, 2009) or social stress (Foster et al., 2006; Tamashiro et al., 2006) become hyperphagic in a high caloric dietary environment. A smaller number of studies in female rodents also indicate that stressor exposure promotes food intake and visceral obesity (Bartness, 1996; Solomon et al., 2011). Although ovarian estradiol is known to reduce food intake (Wade and Schneider, 1992) by limiting the size of a meal (Asarian and Geary, 2006), its effect on stressed-induced consumption of calorically dense diets is unknown. For example, a recent study in premenopausal women reported that subjects expressing high levels of stress engage in more emotional feeding and show a blunted response in cortisol to an acute stressor (Tomiyama et al., 2011). However, it is unclear how ovarian cycle stage affected this pattern. Our model using ovariectomized rhesus monkeys holds the promise of determining how replacement therapy with estradiol may alter the social status

differences in consumption of calorically dense diets. Because estradiol exacerbates the loss of glucocorticoid negative feedback in subordinate females (Wilson et al., 2005) and increased exposure to glucocorticoid is a key signal in the emergence of stress eating (Dallman et al., 2007), one might predict that the effects of estradiol limiting food intake will be lost in the face of a chronic stressor.

Although we did not see a reduction in LHPA activity following acute stress in subordinate or dominant females during the choice diet condition, we did observe that the cortisol response to the social separation predicts the subsequent intake of the HFSD and not LCD independent of social status. These data presented in the current study parallel findings in women indicating that responses to acute stressors may influence subsequent eating behavior by altering food intake and preference for calorically dense food (Adam and Epel, 2007; Appelhans et al., 2010; Epel et al., 2004; Epel et al., 2001). What our data show is that the chronic stress of subordination promotes persistent, increased caloric intake in a rich dietary environment but that the cortisol response to an acute stressor, regardless of a chronic stress background, predicts the subsequent short term intake of a calorically dense but not a lower caloric diet.

Evidence suggests that changes in dopamine (DA) activity is a potential mechanism linking stress to comfort food ingestion (Bassareo and Di Chiara, 1999; Martel and Fantino, 1996a, b; Pelchat, 2002; Rada et al., 2005) as chronic psychosocial stress exposure reduces DA function, characterized by reduced DA D2 receptor (D2R) availability that is associated with anhedonia and increased susceptibility to addiction (Anisman and Matheson, 2005; Harfstrand et al., 1986; Izzo et al., 2005; Koob and Kreek, 2007; Lucas et al., 2004; Macey et al., 2000; Sauvage and Steckler, 2001;

Swanson et al., 1983). Indeed, the imposition of social subordination in macaques leads to a reduction of D2R binding potential assessed by PET in striatal regions (Grant et al., 1998; Morgan et al., 2002) and increased cocaine self-administration (Morgan et al., 2002). This "reward deficiency syndrome", characterized by reduced DA activity (Blum et al., 1996), is both predictive of an addictive phenotype (Volkow et al., 2003; Volkow and Wise, 2005) and observed in obesity (Wang et al., 2001). Therefore, it is hypothesized that a biobehavioral strategy to activate DA pathways compromised by stress is to consume a HFSD, thereby increasing levels of DA in the nucleus accumbens (Bassareo and Di Chiara, 1999), a finding not seen when consuming a LCD or palatable food devoid of calories such as saccharin (Bassareo and Di Chiara, 1999; Blackburn et al., 1986; Marinelli et al., 2006; Small et al., 2003). What is compelling, however, are data showing that consuming a HFSD, independent of stress, produces deficits in mesolimbic D2R availability (Geiger et al., 2009; Johnson and Kenny, 2010; Lee et al., 2010). Because both insulin and leptin can reduce the rewarding value of food (Berthoud et al., 2011; Lustig, 2006; Shizgal et al., 2001), possibly by attenuating DA release (Krugel et al., 2003) and/or increasing D2R binding in these reward pathways (Pfaffly et al., 2010), the insulin and leptin insensitivity that the emergences as obesity develops could account for this diet-induced suppression of mesolimbic D2R availability. Together, these data suggest that psychosocial stress exacerbates diet-induced reductions in D2R, further sustaining the drive for unhealthy eating and creating a downward spiral often observed in addiction. Indeed, the persistence of increased caloric intake by subordinate females following the removal of the HFSD and re-establishment of a healthier dietary environment may be a

compulsive food-seeking behavior driven by the motivation to engage a hypoactive reward system.

In summary, these data are consistent with previous data showing exposure to psychosocial stress increases consumption of overall calories. In addition, these data suggest that chronic psychosocial stress increases eating of low caloric food choices in the presence of a high caloric food choice. Moreover, results show that stress interacts with a history of HFSD consumption to increase food intake even when high caloric food is no longer available. Although it is well established that exposure to social stressors can act as an environmental trigger to induce alterations in appetite regulation, caloric intake, and body weight gain (Bjorntorp, 2001; Dallman et al., 2005; Rosmond, 2004; Schwartz, 2009; Scott et al., 2008), the present study indicates that stressor exposure is a key explanatory factor for determining how an individual's eating can be modulated immediately and for the long-term by the ingestion of calorically dense diets.

Table 4.1. Mean ± SEM kcal per day consumed for each two-week diet phase during the daytime (0600-1800 h) and nighttime (1800-0600 h) in dominant (n= 15) and subordinate (n= 24) females. An asterisk denotes a main effect of diet order presentation. *[From (Michopoulos et al., 2012b)]*

			No Choice		**Choice**	
Diet Order	**Diet**	**Time**	**Dominant**	**Subordinate**	**Dominant**	**Subordinate**
LCD only First	LCD	Daytime	251 ± 80	226 ± 61	123 ± 43	162 ± 33
		Nighttime	96 ± 42	130 ± 32	28 ± 22	65 ± 17
		Total	348 ± 53	356 ± 40	151 ± 55	227 ± 42
	HFSD	Daytime	-	-	210 ± 122	359 ± 93
		Nighttime	-	-	110 ± 19	108 ± 51
		Total	-	-	320 ± 82	467 ± 63
	Total	Daytime	251 ± 80	226 ± 61	333 ± 82	521 ± 63
		Nighttime	96 ± 42	130 ± 32	138 ± 47	173 ± 34
		Total	348 ± 53	356 ± 40	470 ± 64	694 ± 48
Choice First *	LCD	Daytime	374 ± 80	556 ± 61	172 ± 40	226 ± 33
		Nighttime	106 ± 42	193 ± 32	37 ± 21	103 ± 17
		Total	480 ± 53	749 ± 40	210 ± 52	329 ± 42
	HFSD	Daytime	-	-	272 ± 114	406 ± 93
		Nighttime	-	-	80 ± 63	185 ± 51
		Total	-	-	352 ± 72	591 ± 63
	Total	Daytime	374 ± 80	556 ± 61	445 ± 71	632 ± 63
		Nighttime	106 ± 42	193 ± 32	118 ± 42	288 ± 34
		Total	480 ± 53	749 ± 40	563 ± 59	920 ± 48

Table 4.2. Mean ± SEM measures of body weight and serum leptin, insulin, and glucose concentrations at the end of each two-week diet condition (No choice vs. Choice) for both dominant and. subordinate females. Data are presented by the order of diet condition, whether the choice or the no choice phase came first. Also, shown is the change in the measure from the beginning of each condition to the end of the two-week condition. Asterisks denote the significantly greater increase in leptin levels only in subordinate females during the choice condition. Letters depict main effects of diet and numbers illustrate differences due to a significant diet condition by order of diet presentation interaction. See text for details. *[From (Michopoulos et al., 2012b)]*

			Dominant		**Subordinate**	
Endpoint	**Measure**	**Initial Diet**	**No choice**	**Choice**	**No choice**	**Choice**
Weight (kg)	Final	LCD only	8.74 ± 0.56	8.44 ± 0.54	7.93 ± 0.52	7.62 ± 0.41
		Choice	9.41 ± 0.52	9.68 ± 0.50	7.15 ± 0.43	7.24 ± 0.41
	Change	LCD only	-0.17 ± 0.09 [a]	0.03 ± 0.09 [b]	-0.21 ± 0.07 [a]	-0.23 ± 0.07 [b]
		Choice	-0.21 ± 0.09[a]	0.04 ± 0.09 [b]	-0.13 ± 0.07 [a]	0.11 ± 0.07 [b]
Leptin (ng/ml)	Final	LCD only	17.6 ± 2.65	15.3 ± 3.22	7.59 ± 2.02	10.1 ± 2.46
		Choice	13.5 ± 2.47	11.9 ± 3.01	11.9 ± 2.02	15.4 ± 2.46
	Change	LCD only	2.06 ± 2.78	-2.13 ± 3.40	0.91 ± 2.13	6.84 ± 2.59*
		Choice	5.52 ± 2.60	4.71 ± 3.18	0.51 ± 2.13	9.41 ± 2.59*
Insulin (μU/ml)	Final	LCD only	93.1 ± 24.3	50.6 ± 24.7	76.4 ± 18.6	71.3 ± 18.8
		Choice	77.7 ± 22.8	128 ± 23.1	43.6 ± 18.6	72.5 ± 18.6
	Change	LCD only	-28.0 ± 13.3[1]	25.3 ± 19.0[2]	-11.3 ± 10.2[1]	41.1 ± 14.5[2]
		Choice	19.3 ± 12.5	4.10 ± 17.8	6.13 ± 10.2	13.7 ± 14.5
Glucose (mg/dl)	Final	LCD only	96.7 ± 5.63 [a]	95.2 ± 5.73 [b]	89.6 ± 3.98 [a]	93.1 ± 4.05 [b]
		Choice	90.8 ± 4.88 [a]	108 ± 4.96 [b]	88.5 ± 3.98 [a]	95.8 ± 4.05 [b]
	Change	LCD only	-7.78 ± 6.12	2.83 ± 12.8	-3.74 ± 4.71	11.1 ± 9.75
		Choice	9.34 ± 5.77[2]	-17.4 ±11.9[1]	14.2 ± 4.71[2]	-7.09 ± 9.75[1]

Table 4.3. A summary of major outcome differences between dominant (Dom) and subordinate (Sub) females during the two diet conditions. If a difference is stated between groups or dietary conditions, it was significant ($p < 0.05$). *[From (Michopoulos et al., 2012b)]*

Outcome Measure	Result
Aggression received	Sub receive more aggression
Submission emitted	Sub emit more submissive behaviors
Dex suppression test	Sub have reduced glucocorticoid negative feedback
Post-stressor increase in serum cortisol	Dom and Sub respond similarly and the response is greater during the choice compared to LCD-only condition
Average caloric intake	• During LCD-only with no history of HFSD, Dom and Sub ate a similar number of calories • During the choice condition, Sub ate more calories than did Dom • During the choice both Dom and Sub ate more calories from the HFSD compared to the LCD. However, during this condition Sub ate more LCD than did Dom • If the LCD-only condition followed the choice condition, Sub ate more calories than did Dom • Dom ate a similar number of calories regardless of diet condition • Pre and post dexamethasone cortisol predicted HSFD but not LCD intake • Post stressor cortisol predicted HFSD but not LCD caloric intake
Body weight	• At the initiation of study, Dom weighed more than Sub and this status difference was maintained throughout each 2 week diet condition • Despite increased caloric intake during the choice condition, Sub did not gain more weight than Dom
Serum leptin	Serum leptin increased significantly during choice diet condition in Sub but not Dom females
Serum insulin	• If the LCD-only condition was first, serum insulin increased significantly during the choice condition • If the choice condition was first, the increase in serum insulin was similar between both diet conditions • Although the increase was greater in Sub during the choice condition, the difference was not significant
Serum glucose	Serum glucose was higher at the end of the choice condition

Figure 4.1. (A) Mean ± SEM rates (per 30 min) of aggressive behavior received and submission behavior emitted by females at each social dominance rank. Rates of aggression received and submission emitted were higher in animals categorized as subordinate females (ranks 3 – 5) compared with those categorized as dominant (ranks 1 and 2). (B) Mean ± SEM suppression in cortisol levels following dexamethasone administration in dominant (closed bars) and subordinate (open bars) females. Reduced suppression at 0800 by subordinates (*P < 0.05) reflects diminished glucocorticoid negative feedback. *[From (Michopoulos et al., 2012b)]*

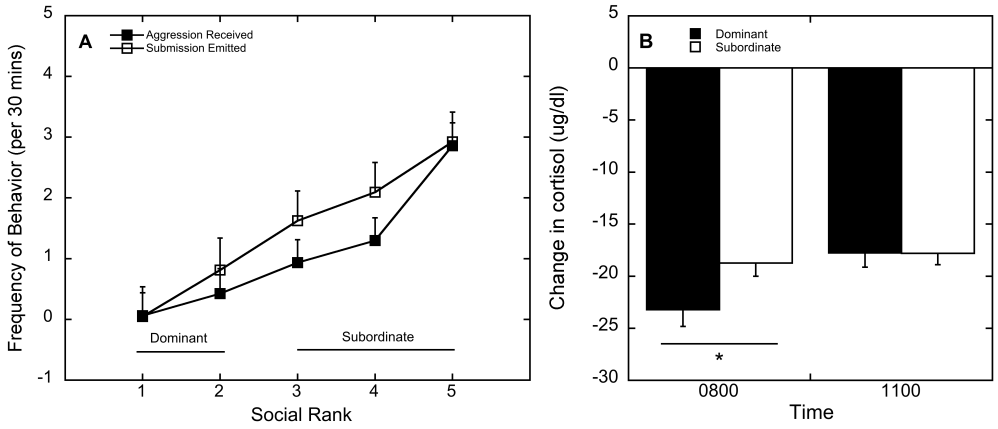


Figure 4.2. Mean ± SEM daily (24-h) caloric intake in dominant and subordinate females by diet condition (choice vs. no choice). Open bars represent intake of the low calorie diet (LCD) and closed bars indicate caloric intake of the high fat and sugar diet (HFSD) diet. Asterisks (*) denote overall preference of the HFSD over the LCD during the choice condition. Different letters (a, b) above each of the four group – diet conditions indicate caloric intake was significantly different ($p < 0.05$) based on post hoc tests. Overall caloric intake and intake of the LCD was significantly increased during the choice condition in subordinate compared to dominant females ($p < 0.05$). *[From (Michopoulos et al., 2012b)]*

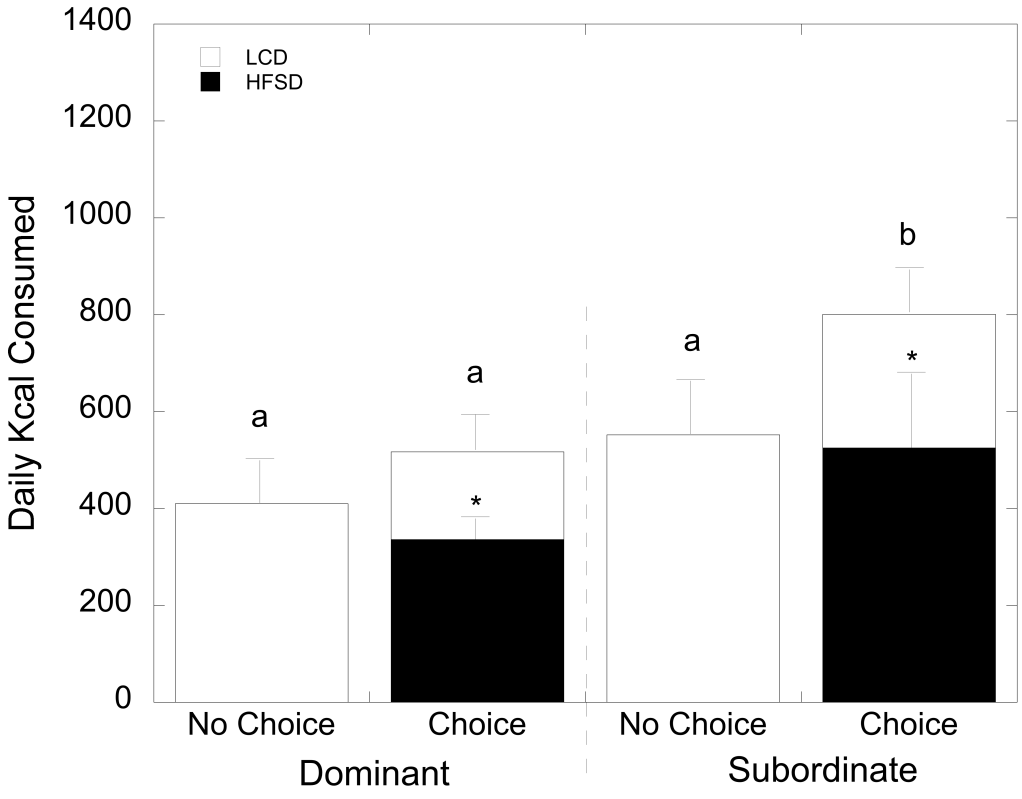


Figure 4.3. Mean ± SEM daily (24-h) caloric intake in dominant (A) and subordinate (B) females by diet condition and order of diet condition. Open bars represent intake of the low calorie diet (LCD) and closed bars indicate caloric intake of the high fat and sugar diet (HFSD). Asterisks (*) denote overall preference of the HFSD over the LCD during the choice condition ($p < 0.05$). Subordinate animals consumed more overall calories when the choice condition preceded the no choice condition (#) whereas order of diet condition did not affect caloric intake in dominant animals. Letters denote significant ($p < 0.05$) differences in intake during each diet condition in subordinate females due to order of diet presentation. *[From (Michopoulos et al., 2012b)]*

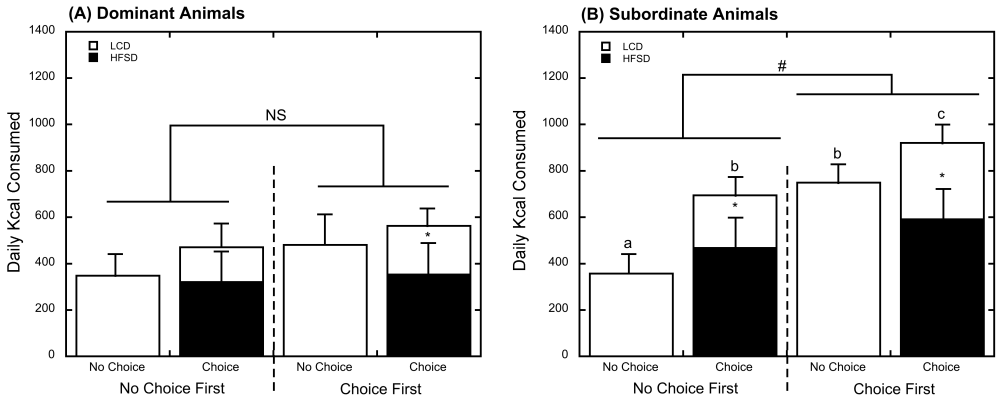


Figure 4.4. Mean ± SEM increase in cortisol levels due to social separation stressor test during the no choice (open bars) and choice (closed bars) diet conditions broken down by social status (dominant vs. subordinate) and order of diet presentation. *p < 0.01. *[From (Michopoulos et al., 2012b)]*

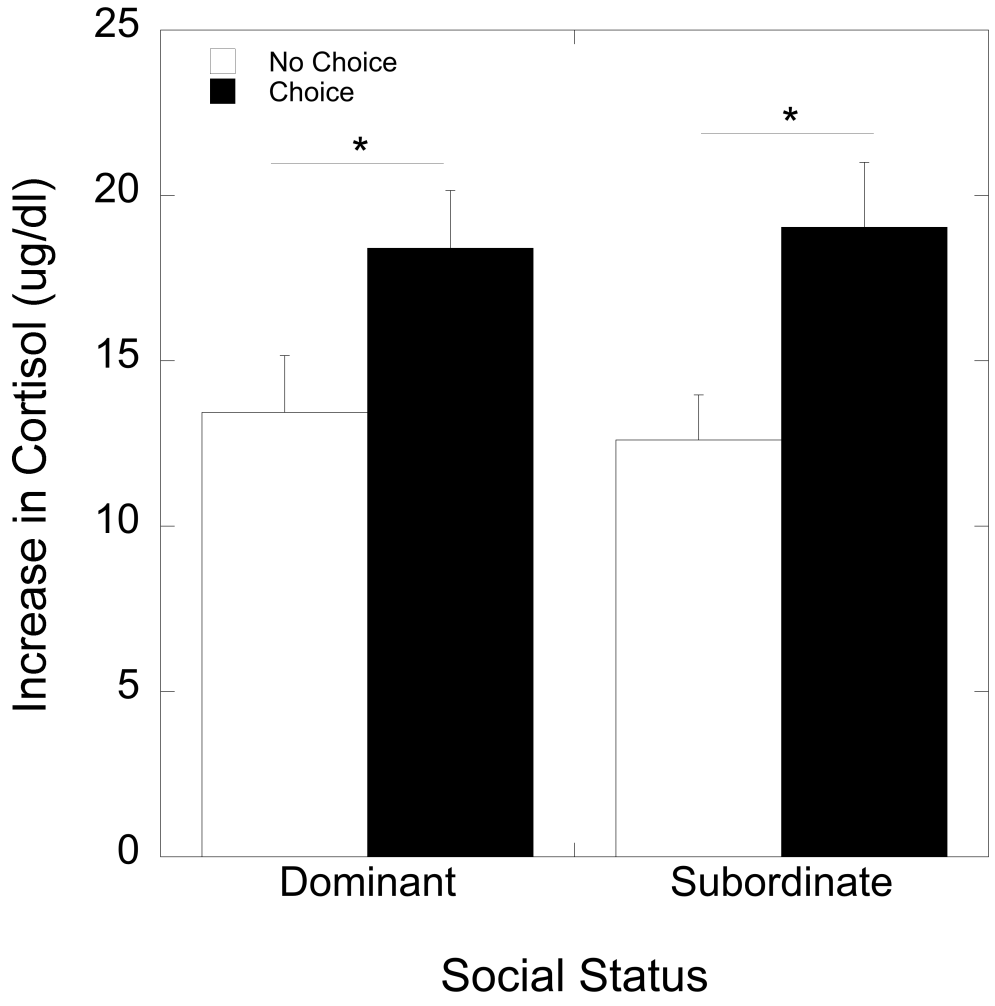
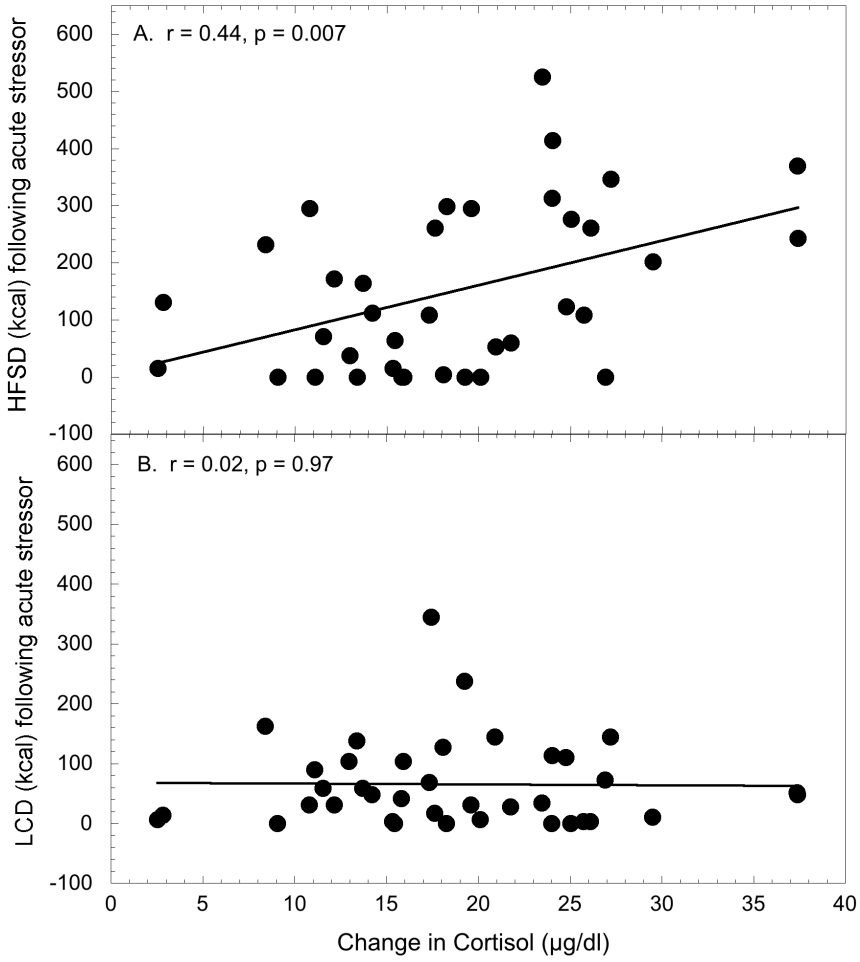


Figure 4.5. Simple linear regression using the increase in cortisol following the social separation stressor to predict consumption of high fat and sugar diet (HFSD; panel A) and low calorie diet (LCD; panel B) in the 9-hr period following the stressor in all animals. Two animals whose intake of the HFSD exceeded 2 standard deviations from the group mean were excluded. Not shown is the non-significant regression of the change in cortisol following the social separation predicting caloric intake of the LCD during the no choice condition ($r_{35} = 0.06$, $p = 0.70$). *[From (Michopoulos et al., 2012b)]*



CHAPTER FIVE:

SOCIAL SUBORDINATION RESULTS IN THE IMPAIRMENT OF REPRODUCTIVE FUNCTION IN FEMALE RHESUS MONKEYS

5.1 Abstract

Psychosocial factors compromise reproduction by inducing menstrual cycle disturbances in mammals, including women and macaques. While reproductive dysfunction is characteristic of subordinate social status in macaque females, the mechanism responsible has not been elucidated. The etiology of reproductive compromise could stem from the altered activity of the LHPA axis characteristic of subordinate females or from the decreased body weight and attenuated feeding in subordinates on a standard monkey chow. The following chapter presents a series of experiments designed to assess the potential mechanisms responsible for the higher frequency of anovulation in subordinate females and to determine whether the polymorphism in the gene encoding the serotonin transporter (5HTT) increases individual vulnerability to stress-induced infertility. We show that subordination enhances estradiol (E2) negative feedback inhibition of luteinizing hormone (LH) and that a pharmacological elevation of serum cortisol potentiates E2 negative feedback inhibition of LH in all females. However, our data show that E2 positive feedback of LH is not affected by social status and 5HTT genotype. We conclude that the increased frequency of reproductive compromise characteristic of subordinate female macaques is likely due to hypersensitivity to E2 negative feedback inhibition of LH that results in low levels of both E2 and LH and thus impedes the ability for subordinate animals to mount an LH surge necessary for ovulation. We also show that while a two-week exposure to a high fat sugar diet (HFSD) results in increased caloric intake, it does not affect E2 negative feedback of LH in subordinate females. Thus, while it is possible reproductive compromise in socially subordinate females is not due to disrupted energy regulation, a

longer period of HFSD exposure and increased caloric intake is necessary to induce biologically relevant changes in metabolism that could mitigate the effects of psychosocial stress exposure on reproductive physiology in subordinates.

5.2 Introduction

Fertility is often punctuated by reproductive deficits in female mammals (Wade and Jones, 2004), including macaque species (Chrousos et al., 1998; Ferin, 1999; Matsumoto et al., 1979; Wilks et al., 1976, 1977; Williams et al., 1982). Luteal deficiencies are a result of inadequate gonadotropin release result in both monkeys and women (Wilks et al., 1976). Furthermore, like other cercopithecine primates, macaque species show anovulatory menstrual cycles as defined by an absence of a sustained elevation in serum progesterone levels above 1 ng/ml (Adams et al., 1985; Aso et al., 1977; Mori et al., 1973; Wilks et al., 1977), highlighting the importance of optimal gonadotropin control of ovarian folliculogenesis for ovulation (Wilks et al., 1977). While reduced reproductive success in subordinate female macaques (Wilson et al., 1978) is most often characterized by extended periods of anovulation and increased incidence of short luteal phase cycles (Adams et al 1985; Pope et al 1986; Shively et al 1997; Kaplan et al 2010), the mechanism responsible for this phenotype has not been elucidated.

5.2.1 Regulation of reproductive axis in females

The ovarian cycle in female macaques is similar to that of women, consisting of a follicular, preovulatory and luteal phase (Hodgen, 1989). The follicular phase is characterized by the maturation of single follicle, containing a single ovum, and increasing levels of estradiol (E2) (Hodgen, 1989). E2 levels peak at the time of ovulation when the ovum is released from the follicle into the fallopian tubes for potential fertilization (Irianni and Hodgen, 1992). Ovulation is followed by the luteal phase, when

the follicle becomes the progesterone (P4) secreting corpus luteum. The uterine lining thickens during the follicular phase and is shed following the luteal phase if fertilization and implantation does not occur, resulting in menstruation (Hodgen, 1989). The timing and orchestration of these physiological changes critical for the maintenance of fertility in females is controlled by the hypothalamic – pituitary – gonadal (HPG) axis.

A collection of gonadotropin-releasing hormone (GnRH)-secreting neurons localized in the mediobasal hypothalamus releases GnRH in a pulsatile fashion into the median eminence (Kallo et al., 2011). GnRH induces the synthesis and release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary into the blood stream where they bind to receptors on thecal and granulosa cells of the ovary to stimulate steroidogenesis (Yen and Jaffe, 1991). In females, LH is important for the synthesis of androstenedione that is transported from thecal to granulosa cells within the follicle (Yen and Jaffe, 1991). Androstenedione is enzymatically converted to testosterone and aromatized to E2 under the control of FSH (Yen and Jaffe, 1991). During the early follicular phase, E2 secretion progressively increases culminating in a surge of gonadotropin release that ruptures the Graafian follicle to release the egg and stimulates the transformation of granulosa cells to luteal cells under the control of an increased expression of LH receptors and a change in steroidogenic pathways towards the production of progesterone, which, in part, prepares the endometrium in the event the egg is fertilized (Yen and Jaffe, 1991).

Synchronization of GnRH firing within the hypothalamus is critical for producing the appropriate signal to drive the ovarian cycle. *In vitro* studies show that GnRH is released from the hypothalamus at a pulse every 25 minutes, which induce simultaneous

release of LH from the pituitary. Thus, measuring LH in the periphery is used as a surrogate measure of central GnRH activity (Clarke and Cummins, 1982; Ordog et al., 1997). Lesions of the GnRH cell bodies and replacement of GnRH in ovariectomized monkeys at a rate of one pulse per hour reinstates both LH and FSH secretion whereas continuous infusion of GnRH results in suppression of LH and FSH (Belchetz et al., 1978; Wildt et al., 1981) due to a downregulation of pituitary type 1 GnRH receptors (Naftolin et al., 2007; Pau et al., 1993). The control of this crucial GnRH synchronization is modulated by glia cells (Witkin et al., 1997), opioids such as β-endorphin (Sapolsky and Krey, 1988) and neurosteroids such as allopregnanolone (Meczekalski et al., 2000; Rasmussen, 1986; Terasawa, 1995; Thind and Goldsmith, 1989).

The regulation of the HPG axis at both the level of the hypothalamus and the pituitary is contingent upon the ability of E2 to induce negative and positive feedback on GnRH and, thus, LH and FSH secretion. Data show that upon removal of E2 by ovariectomy in female monkeys, LH pulse frequency approaches one pulse per hour. However, when E2 levels are restored to serum concentrations similar to those observed during the early follicular and luteal levels, LH pulse frequency is reduced (Condon et al., 1988; Yamaji et al., 1972; Yen and Tsai, 1971b). These observations are confirmed by studying gonadotropin pulse patterns in gonadally intact women (Berga and Naftolin, 2012). However, as the cycle advances towards ovulation, serum E2 levels steadily rise and induce a surge of LH that stimulates ovulation (Condon et al., 1988; Karsch et al., 1973; Yen and Tsai, 1971a). This dual negative and positive feedback action of E2 on GnRH and gonadotropin secretion is dependent on the dose and duration of E2 exposure

on GnRH neurons in the hypothalamus (Silverman et al., 1979; Witkin et al., 1991) and gonadotropes in the pituitary (Kallo et al., 2011; Naftolin et al., 2007; Pau et al., 1993).

Studies employing the use of estrogen receptor (ER) knockout mouse models have shown that while ERβ minimally influences the ability of E2 to suppress LH levels, it is the ERα subtype that mediates E2's ability to feedback negatively on the HPG axis (Dorling et al., 2003). While GnRH neurons do not express ERα (Sullivan et al., 1995; Watson et al., 1992) these neurons do express ERβ (Herbison and Pape, 2001; Hrabovszky et al., 2000). Recent evidence from rhesus monkeys indicates that E2 induces rapid excitation of GnRH neurons that occurs at the cell membrane and results in rapid changes in the oscillation and sychronization of the GnRH cell bodies in the hypothalamus (Terasawa et al., 2010). This non-classical action of E2 in combination with the classical actions of E2 via activation of ERα and ERβ modulate the activity of the GnRH neurons to control the release of GnRH over the course of the ovarian cycle. Importantly, the differential ability for E2 actions to modulate GnRH and LH release might be linked to neuroanatomical interactions with other neurochemical systems that are important for the regulation of GnRH secretion.

One possible switch from negative to positive feedback of E2 on GnRH and LH secretion is modulated by kisspeptin (Hameed et al., 2011). Kisspeptin is localized to the anteroventral periventricular nucleus (AVPV) and arcuate nucleus of the hypothalamus in rodents (Gottsch et al., 2004) and the infundibular nucleus in primates (Rometo et al., 2007). Kisspeptin neurons are in close proximity with GnRH cell bodies that express GPR54, the kisspeptin receptor, in rodents and in primates (Clarkson et al., 2008; Silverman et al., 1977) and act to facilitate E2 negative and positive feedback of LH at

each of these sites, respectively (Adachi et al., 2007; Wiegand and Terasawa, 1982). Kisspeptin in the AVPV of rats is critical for the stimulation of LH secretion by increasing the expression of GnRH (Adachi et al., 2007; Kinoshita et al., 2005; Navarro et al., 2005; Smith et al., 2005). Importantly these kisspeptin neurons respond to E2 as peripheral administration of an ERα antagonist blocks the preovulatory LH surge (Roa et al., 2008; Spratt et al., 1993).

While kisspeptin is generally described as an excitatory signal to GnRH neurons and important for the induction of an LH surge, kisspeptin works in concert with galanin and neurokinin to induce GnRH secretion (Kallo et al., 2011; Navarro et al., 2009). It is also important to note that inhibitory systems are also critical for the regulation of the reproductive axis, such as the γ-aminobutyric acid (GABA) system. Indeed, the GABAergic system modulates GnRH, and thus LH, secretion (Lagrange et al., 1995; Leranth et al., 1985). The ability of E2 to sequentially inhibit and then stimulate GnRH secretion over the course of the ovarian cycle is also linked to the biphasic effects of E2 on GABAergic neuron that modulate the activity of GnRH neurons (Wagner et al., 2001). Early during E2 exposure (first 42 hours in rodents), E2 decreases the auto-inhibition of GABAergic neurons, resulting in an increase of GABAergic inhibition in the medial basal hypothalamus that is associated with decreased GnRH drive and E2 negative feedback (Wagner et al., 2001). Beyond this early 42 hour exposure, GABAergic auto-inhibition is unaffected by E2, and E2 decreases the expression of genes that encode enzymes that are necessary for GABA synthesis (GAD65 and GAD67) that reduces GABAergic inhibition on downstream targets, such as the GnRH neurons (Wagner et al., 2001).

Thus, the control of the HPG axis is a tightly regulated physiological system whose activity is altered by changing feedback mechanisms that are crucial for the progression of follicular development, ovulation, and if fertilization occurs, implantation. The location of the GnRH neurons in the hypothalamus makes it aptly positioned to receive inputs from an array of systems that can influence reproductive physiology. The colocalization of neuromodulatory systems and inputs from the cortex and peripheral organs qualifies the hypothalamus as the integration center of all relevant internal and external cues that are important in controlling the ovarian cycle and reproduction. A number of external factors can impinge upon the normal functioning of the HPG axis.

5.2.2 Interaction between HPG and LHPA axes

Stress-induced anovulation (SIA) in women, more commonly referred to as functional hypothalamic amenorrhea (FHA), not only causes infertility, but also increases vulnerability to other diseases of aging and chronic stress exposure (Berga and Loucks, 2005, 2006; Kaplan and Manuck, 2004; Marcus et al., 2001). Importantly, FHA is a psychoneuroendocrinologic condition that is characterized by hypogonadism resulting from decreased drive of the hypothalamic GnRH neurons. Women with FHA are hypercortisolemic; not only are levels of circulating cortisol throughout the day higher in women with FHA in comparison with eumenorrheic women (Berga et al., 1989; Suh et al., 1988), but central levels of cortisol in CSF are also elevated in FHA women when compared to eumenorrheic women (Brundu et al., 2006).

Studies in animal models have been crucial for elucidating the role of the LHPA axis in modulating the activity of the HPG axis. Activity of the LHPA axis directly

modulates GnRH pulsatility via both cortisol and CRH. Studies in ewes show that cortisol reduces GnRH and LH pulse frequency (Oakley et al., 2008), effects that are dependent upon the background hormonal milieu as it is only seen when follicular levels of estradiol and progesterone are present (Breen et al., 2005; Oakley et al., 2009). These inhibitory effects of cortisol on GnRH and LH are attenuated when type II glucocorticoid receptors are antagonized (Breen et al., 2007). Furthermore, the central effects of CRH can influence the GnRH secretion from the hypothalamus. CRH induces release of β-endorphin, cleaved from proopiomelanocortin (POMC), from arcuate hypothalamic cells, which inhibits GnRH pulsatility (Gindoff and Ferin, 1987; Sapolsky and Krey, 1988). Administration of opioid antagonists, such as naloxone, is capable of increasing LH levels in some women with FHA (Khoury et al., 1987; Quigley and Yen, 1980). Additionally, central CRH administration inhibits the secretion of LH in female rats (Petraglia et al., 1987) and monkeys (Olster and Ferin, 1987), and the continuous expression of CRH in the central nucleus of the amygdala in female rats disrupts estrous cycles (Keen-Rhinehart et al., 2009). These data suggest that CRH acts directly on GnRH cell bodies that project to the median eminence (Dudas and Merchenthaler, 2002; MacLusky et al., 1988).

Furthermore, *in vitro* studies indicate that cortisol acting through glucocorticoid receptors (GRs) rapidly inhibits LH pulsatility directly at the level of the pituitary (Breen et al., 2008), a finding that has been extended to *in vivo* studies in ewes (Breen et al., 2008; Breen et al., 2007) and in women (Saketos et al., 1993). Cortisol diminishes pituitary sensitivity to GnRH that results in LH inhibition (Breen et al., 2008; Breen and Karsch, 2006). It is important to note that GRs are also expressed in the granulosa cells

of ovary (Schreiber et al., 1982) and activation of ovarian GRs results in decreased responsiveness to gonadotropins (Hsueh and Erickson, 1978; Michael et al., 1993). This direct effect of cortisol on the ovary suggests that glucocorticoids could inhibit secretion of E2, thus delaying the LH surge or inhibiting the production of a LH surge (Breen et al., 2008). Taken together, the LHPA axis acts at all three levels of the HPG axis to disrupt its function and induce reproductive compromise.

5.2.3 Metabolic signals modulate reproductive function

In addition to stress-induced disruptions, changes in metabolic signals can also affect reproductive function. States of negative energy balance result in the cessation of reproduction function and lead to reversible infertility in female mammals (Wade and Jones, 2004). Such states of infertility in women result from excessive exercise and decreased food intake, as seen in anorexia (Marcus et al., 2001). Women with FHA have decreased levels of leptin reflecting decreased adiposity (Laughlin et al., 1998; Welt et al., 2004) and increased levels of ghrelin, an orexigenic peptide from the stomach, (Schneider and Warren, 2006), observations consistent with data from women with eating disorders and/or deficits in energy balance (Leidy et al., 2004; Tanaka et al., 2002; Tolle et al., 2003).

Observations in rodents and monkeys indicate metabolic signals are important modulators of GnRH and LH secretion. Leptin stimulates GnRH and LH secretion at the level of the hypothalamus in rodents (Watanobe, 2002) and in monkeys (Finn et al., 1998), an effect modulated by kisspeptin (Castellano et al., 2005; Smith et al., 2006a; Smith et al., 2006b). Central ghrelin administration suppresses LH levels in female rats

(Fernandez-Fernandez et al., 2004; Furuta et al., 2001) and peripheral ghrelin administration decreases LH pulse frequency in female rhesus monkeys (Vulliemoz et al., 2004). Central infusion of AgRP (agouti-related protein), an orexigenic signal from the hypothalamus, also suppresses LH pulsatility in female macaques (Vulliemoz et al., 2005). Leptin, ghrelin and AgRP all modulate the hypothalamic neuropeptide Y (NPY) system (Furman and Wade, 2007). NPY is an orexigenic peptide whose expression is increased in states of negative energy balance (Furman and Wade, 2007). NPY neurons synapse directly on GnRH cell bodies (Turi et al., 2003) and modulate the activity of GnRH neurons (Terasawa, 1994, 1995). The destruction of NPY cell bodies results in a release of LH inhibition (I'Anson et al., 2003) whereas central administration of NPY inhibits LH secretion (Raposinho et al., 1999; Tsukamura et al., 1994).

Importantly, NPY, and thus the other metabolic signals, can also affect the activity of GnRH neurons via activation of the stress axis (Williams et al., 1990). Release of NPY induces subsequent release of CRH (Wahlestedt et al., 1987) that then acts to inhibit the activity of GnRH neurons (MacLusky et al., 1988). The capacity of CRH to mediate the effect of metabolic signals on the reproductive axis is also evident by the ability of a CRH receptor antagonist to block ghrelin's ability to suppress LH levels in monkeys (Vulliemoz et al., 2008). Urocortins also play an important role in energy balance and metabolism because they act to reduce food intake via CRH receptors (Richard et al., 2002). Together, these data suggest that metabolic signals converge upon the LHPA axis, indicating that concomitant metabolic and psychogenic stress synergize to suppress activity of the HPG axis. Indeed, macaque females subjected to both a

metabolic and psychogenic stressor show increased incidence of ovulatory disruptions compared to animals that received either stressor by itself (Williams et al., 2007).

5.2.4 *SLC6A4* Polymorphism and increased vulnerability to stress-induced adverse health outcomes

While social subordination produces reproductive deficits ranging from the timing of puberty through adult fertility, some females are more compromised than others (Adams et al., 1985; Kaplan et al., 1984; Kaplan et al., 2010; Pope et al., 1986; Shively et al., 1997b; Walker et al., 1983) suggesting individual differences in stress responsivity determine the ultimate reproductive phenotype. Previous studies show that female rhesus monkeys that are most reactive to stress are characterized by diminished serotonergic (5HT) activity, evidenced by reduced response to fenfluramine (Bethea et al., 2005a), reduced 5HT transporter (5HTT) and monoamine oxidase A (MAO-A) gene expression in the dorsal raphe (Bethea et al., 2005b), reduced 5HT receptor expression in the paraventricular nucleus of hypothalamus (PVN) and infundibulum (Centeno et al., 2007a), and greater CRH expression in limbic-hypothalamic regions (Centeno et al., 2007b). Diminished 5HT function is associated with trait-like disinhibition of behavior in response to social adversity, including increased impulsivity and aggression as well as poor regulation of affect (Carver et al., 2008; Higley and Linnoila, 1997). Thus, in addition to these differences in behavior that emerge in response to social challenges, reduced 5HT activity may predispose females to reproductive compromise.

Polymorphisms in the gene encoding the 5HT transporter (5HTT; *SLC6A4*) produce differences in 5HT function (Bennett et al., 2002; Lesch et al., 1997). The short

promoter length allele interacts with social adversity to increase the incidence of affective disorders in people (Caspi et al., 2003; Lesch et al., 1996; Melke et al., 2001; Veenstra-VanderWeele et al., 2000) and increases emotionality (Bethea et al., 2004; Champoux et al., 2002), stress hormone responsivity (Barr et al., 2004b) and behavioral reactivity in rhesus monkeys (Jarrell et al., 2008). Although the contribution of 5HTT polymorphisms to a reproductive phenotype has produced equivocal results (Bethea et al., 2005a; Hoffman et al., 2007), it is possible that the 5HTT short promoter length genotype may predispose females to stress-induced reproductive compromise. Indeed, the lab has shown previously that subordinate female rhesus monkeys with the s-variant 5HTT genotype are more likely to have delayed puberty (Wilson and Kinkead, 2008). Whether polymorphisms in the gene encoding the 5HTT influences affects of social subordination on the regulation of reproduction in adult females is not known.

5.2.5 Overall rationale

Because social subordination in female rhesus monkeys results in increased frequency of ovulatory deficits and anovulation (Kaplan and Manuck, 2008), it becomes imperative to ask how exposure to psychosocial stress compromises reproduction in subordinate females. Consequently, my goal was to define possible mechanisms to account for reproductive compromise in subordinate females and whether identified mechanisms were exacerbated by 5HTT polymorphisms. Specifically, I addressed whether social subordination alters E2 negative and positive feedback of LH and whether signals from the LHPA axis play a role in altering reproductive physiology in subordinate females. Furthermore, because subordinate females macaques fed the typical laboratory

diet have lower body weights, less body fat, and reduced serum concentrations of leptin in comparison to dominant females (Jarrell et al., 2008), we assessed whether a diet induced increase in caloric intake would affect E2 negative feedback inhibition of LH in subordinate females.

5.3 The effects of social subordination and the polymorphism in the gene encoding 5HTT on estradiol inhibition of luteinizing hormone [All text and figures from (Michopoulos et al., 2009a)]

5.3.1 Introduction

The present study was designed to test the hypothesis that social subordination would significantly attenuate LH secretion in adult female rhesus monkeys and that any such effect would be exacerbated by the presence of the short promoter length allele in the 5HTT gene. Furthermore, because stress-induced changes in reproductive hormones may be dependent on the presence of estradiol (Judd et al., 1989; Oakley et al., 2008; Pierce et al., 2008b), the study also tested the hypothesis that subordinate females would be hypersensitive to estradiol negative feedback inhibition of LH. Finally, an unexpected outcome of a primary pharmacologic manipulation (exposure to a corticotropin releasing hormone (CRH) antagonist [CRHA]) resulted in a paradoxical increase in cortisol release, allowing us to assess also the differential effect of relative hypercortisolemia on LH suppression in relation to both social status and 5HTT genotype.

5.3.2 Materials and methods

Subjects. Subjects were 37 ovariectomized adult female rhesus monkeys that were housed in one of eight small social groups at the YNPRC Field Station (n = 4 or 5 per group) without adult males. The social status by genotype groupings were: 8 dominant l/l; 8 dominant s-variant; 9 subordinate l/l, and 12 subordinate s-variant. Groups had been formed for 18 months prior to the start of the present study and were ovariectomized 6 months prior to the formation of these groups. This study was performed in the breeding season between the months of January and March.

Treatment Conditions. All animals were studied in each of four treatment conditions. Each condition lasted 5 days and was separated by a two week, no treatment washout period. All of the females in a specific social group received the same treatment and the order of the treatments was counterbalanced across the groups. The four treatments were 1) control (Con); 2) estradiol (E2) replacement; 3) CRH receptor antagonist (CRHA); and 4) E2 plus CRHA. Saline was administered at 0830 hours every morning for five consecutive days as the Con condition. E2 replacement was achieved by implanting E2 filled Silastic capsules subcutaneously as described previously (Mook et al., 2005). Analysis of selected samples (day 4 and Day 7) during E2 replacement versus no E2 replacement conditions indicated hormone replacement achieved mid-follicular phase concentrations (66.7 ± 2.1 vs. < 5.0 pg/ml). Capsules were implanted three days prior to the initiation of data collection and removed immediately following the end of the phase.

The CRHA used was the CRH type 1 receptor antagonist CP154526 (Pfizer, Groton CT). This CRHA and its close analogue, antalarmin were developed to penetrate

the blood brain barrier and antagonize peripheral and central CRH type 1 receptors as a possible treatment for stress-induced disorders (Keller et al., 2002; Seymour et al., 2003). Based on the existing literature in monkeys using antalarmin (Ayala et al., 2004; Broadbear et al., 2004; French et al., 2007; Habib et al., 2000), we chose to administer a dose of 10 mg/kg daily (sc) for 5 consecutive days at 0830 hours with the expectation that it would attenuate cortisol secretion. However, as described in ***5.1.2 Results*** and reported previously (Broadbear et al., 2004), this dose paradoxically stimulated cortisol secretion, allowing us to evaluate the impact of an increase in cortisol on LH secretion in female monkeys.

Outcome measures. The social housing design precluded the use of indwelling catheters for the frequent sampling of cortisol and LH in peripheral blood. Consequently we measured both cortisol and LH in single morning serum samples obtained 30 minutes following the saline or CRHA injection (i.e., at 0900 hours) on days 2 – 5 of each phase. A similar approach has been used to quantify the seasonal (Walker et al., 1984; Wilson et al., 1987) and developmental (Wilson et al., 2004a; Wilson et al., 1986) regulation of LH in socially housed rhesus monkeys. Because a single morning cortisol sample does not reliably discriminate dominant versus subordinate females (Gust et al., 1993; Jarrell et al., 2008; Stavisky et al., 2001), the intent of the cortisol measurements were used to assess treatment effects of E2 and the CRHA rather than consequences of social subordination. Finally, using an established ethogram, aggressive and submissive behavior for each female was recorded for 30 minutes five hours after the saline or CRHA injection on the five consecutive days of each phase to assess social interactions and verify psychogenic stress (Jarrell et al., 2008).

Statistical analyses. Data were summarized as mean ± SEM. The main effects of status (dominant vs. subordinate), 5HTT genotype (l/l vs. s-variant), E2 treatment (placebo vs. hormone), CRHA treatment (saline vs. drug), and time on treatment (days 1 or 2 through 5), as well as their interactions on hormonal data were analyzed with analysis of variance for repeated measures. Because the behavioral data were not normally distributed, these data were analyzed with nonparametric statistics using Mann-Whitney Tests for two independent samples (e.g., dominant vs. subordinate, l/l vs s-variant, or subordinate l/l vs subordinate s-variant), the Kruskal-Wallis test for more than two independent samples (e.g., ranks 1 – 5 or each social status category at each genotype), the Wilcoxin test for two related samples (e.g., subordinates at two treatment conditions), and the Friedman test for more than two related samples (e.g., comparison of four treatment conditions). To support the categorization of monkeys ranked 1 and 2 in their groups as dominant and those ranked 3 – 5 as subordinate, analysis of agonistic behavior was also performed on a female's individual rank. Two-tailed tests with a $p \leq 0.05$ were considered significant.

5.3.3 Results

Behavioral Characteristics. Table 5.1 shows hourly rates, collapsed across the four treatment conditions, for aggressive and submissive behavior directed toward others as a function of individual dominance ranks and social status categories. Rates of aggression directed towards others varied significantly across the five dominance ranks ($p < 0.01$). When animals were categorized as dominant or subordinate, rates of aggression were significantly higher in dominant females ($p < 0.01$). Furthermore,

females with an s-variant 5HTT genotype had significantly higher rates of aggressive behavior compared to females with an l/l genotype ($p = 0.05$). Indeed, rates of aggression directed towards other was significantly higher in dominant s-variant compared to dominant l/l females ($p = 0.03$). Rates of submissive behavior emitted by animals increased significantly with lower dominance ranks ($p < 0.01$). Thus, higher rates of submissive behavior were also evident in females considered subordinate versus those categorized as dominant ($p < 0.01$). In addition, females with an s-variant genotype emitted significantly higher rates of submissive behavior ($p = 0.01$) and subordinates with the s-variant genotype had higher rates of submissive behavior compared with subordinate, l/l females ($p = 0.01$). Rates of aggression received, reflecting the amount of harassment subordinate monkeys receive from more dominant animals (Figure 5.1), increased significantly with lower dominance ranks ($p < 0.01$) which was again reflected in higher rates for females classified as subordinate as opposed to dominant females ($p < 0.01$). In addition to significantly higher rates of harassment by s-variant females ($p = 0.02$), subordinate s-variant females received more aggression and dominant females than l/l subordinates (Figure 5.1, $p = 0.02$).

Hourly rates of aggression towards others (and its reciprocal, aggression received from others) were significantly increased by E2 (1.30 ± 0.28 vs. 2.96 ± 0.76; $p < 0.01$) but were unaffected by CRHA treatment (2.67 ± 0.74 vs. 1.59 ± 0.31; $p = 0.25$). Hourly rates of submissive behavior towards other was not affected by E2 (1.32 ± 0.35 vs. 1.95 ± 0.43; $p = 0.39$) but was significantly attenuated by CRHA (2.20 ± 0.52 vs. 1.07 ± 0.17; $p < 0.01$). However, the interaction of the CRHA did not significantly attenuate submissive behavior when combined with E2 (2.41 ± 0.96 vs. 1.19 ± 0.39; $p = 0.39$). The

effects of status on agonistic behavior described above were not significantly influenced by the specific treatments ($p > 0.05$). Taken together, these data show that subordinate females are most frequently the recipients of increased aggression from more dominant animals and this pattern of harassment is greatest in females with s-variant genotype. Furthermore, rates of aggression were increased by E2 but not CRHA treatment.

Body Weights. Table 5.2 lists body weights for subjects throughout the four treatment phases. Overall dominant females are significantly heavier than subordinates ($F_{1, 33} = 9.71$, $p < 0.01$). Although females with an l/l 5HTT genotype had higher baseline body weights than those with an s-variant genotype, differences were not significant ($F_{1, 33} = 3.45$, $p = 0.07$). The social status difference in body weight was not significantly modified by genotype ($F_{1, 33} = 1.94$, $p = 0.18$). Treatment with E2 decreased body weights in all females ($F_{1, 33} = 10.26$, $p < 0.01$). Although this effect of E2 was not modified by status ($F_{1, 33} = 0.60$, $p = 0.44$), females with the s-variant genotype lost significantly less weight during the E2 phases than did females with an l/l genotype ($F_{1, 33} = 21.74$, $p < 0.01$). However, this interaction of E2 treatment with genotype was not further modified by status ($F_{1, 33} = 0.27$, $p > 0.61$). Treatment with the CRHA also significantly decreased body weight ($F_{1, 33} = 6.98$, $p = 0.01$) but this effect was not modified by status, genotype, or their interaction ($p > 0.05$). Finally, the combination of E2 and CRHA did not reduce body weights further than each treatment by itself ($F_{1, 33} = 0.01$, $p = 0.95$).

Serum cortisol. As illustrated in Figure 5.2, E2 decreased ($F_{1, 33} = 14.90$, $p < 0.001$) and the CRHA increased ($F_{1, 33} = 12.55$, $p = 0.001$) morning cortisol values in all subjects. Daily cortisol concentrations were consistently lower throughout the week of

E2 treatment compared to placebo ($F_{3, 99} = 0.24$, $p = 0.87$) but did increase progressively throughout the week during the CRHA treatment ($F_{3, 99} = 6.55$, $p < 0.01$). The effect of E2 on cortisol concentrations was not significantly affected by social status ($F_{1, 33} = 2.08$, $p = 0.16$), 5HTT genotype ($F_{1, 33} = 0.01$, $p = 0.96$), or the interaction of status and genotype ($F\ 1_{, 33} < 0.01$, $p = 0.99$). Similarly, the CRHA–induced elevation in cortisol was unaffected by status ($F_{3, 99} = 0.96$, $p = 0.41$), genotype ($F_{3, 99} = 2.22$, $p = 0.90$), or their interaction ($F_{3, 99} = 1.23$, $p = 0.30$).

Serum LH. Serum LH was significantly lower during E2 treatments compared with the non-E2 treatment conditions (3.94 ± 0.17 vs. 6.31 ± 0.28 ng/ml; $F_{1, 33} = 85.16$, $p < 0.01$). Consequently, the effects of status, genotype, and the CRHA treatment were evaluated separately for the non-E2 and E2 conditions. In the absence of E2, serum LH did not vary significantly by time between dominant and subordinate females of either genotype ($F_{3, 99} = 0.99$, $p = 0.73$) so data are collapsed across treatment days (Figure 5.3). As can be seen, there were no differences in morning LH between dominant and subordinate females ($F_{1, 33} = 0.01$, $p = 0.99$) but serum LH was significantly higher in females with an s-variant compared with the l/l 5HTT genotype ($F_{1, 33} = 5.12$, $p = 0.03$). Moreover, in the absence of E2, LH was significantly lower during CRHA treatment ($F_{1, 33} = 3.95$, $p = 0.057$) but this was unaffected by days on treatment ($F_{3, 99} = 1.25$, $p = 0.30$), social status ($F_{1, 33} = 2.14$, $p = 0.16$) or genotype ($F_{1, 33} = 2.33$, $p = 0.15$).

A different pattern emerged during E2 replacement, as morning LH was significantly lower in subordinate (3.51 ± 0.22 ng/ml) compared with dominant females (4.38 ± 0.25 ng/ml; $F_{1, 33} = 6.62$, $p = 0.02$). However, the response in LH to E2 treatment did not vary significantly by 5HTT genotype (l/l: 3.83 ± 0.24; s-variant: 4.05 ±

0.23 ng/ml; $F_{1, 33} = 0.47$, $p = 0.53$) or a status by genotype interaction ($F_{1, 33} = 0.04$, $p = 0.84$). Importantly, this main effect of status varied significantly by day of treatment ($F_{3, 99} = 5.53$, $p < 0.01$), as serum LH was significantly lower in subordinates during the early portion of E2 treatment (days 4 and 5) but not the later portion (days 6 and 7; Figure 5.4).

Treatment with the CRHA, which elevated cortisol levels in all females (Figure 5.3), significantly lowered morning LH when combined with E2 (3.72 ± 0.17 ng/ml) compared with the E2 only condition in all females (4.17 ± 0.22 vs.; $F_{1, 33} = 5.18$, $p = 0.03$, Figure 5.3). This effect of CRHA was consistent across the treatment period, as there was not a significant CRHA by day interaction ($F_{3, 99} = 0.82$, $p = 0.49$). Furthermore, the interaction of status, genotype, and treatment day was not significant ($F_{3, 99} = 0.32$, $p = 0.81$). However, there was a significant status by genotype by CRHA treatment (E2 vs E2 + CRHA) by day interaction ($F_{3, 99} = 2.89$, $p = 0.04$). Post hoc analyses of how each group responded to the treatments indicated subordinate, s-variant females were maximally suppressed by day 5 of treatment during the E2 only phase while dominant, s-variant females were maximally suppressed by day 6. The dominant and subordinate females with the l/l 5HTT genotype both showed a progressive and significant decline in serum LH from days 4 through 7 of E2 only (Figure 5.4, left panel; post hoc tests $p < 0.05$). In contrast, during E2 plus CRHA phase, all females reached nadir concentrations of serum LH by day 6 of treatment (Figure 5.4, right panel; post hoc tests $p < 0.05$).

5.3.4 Discussion

The current study demonstrates that socially subordinate female rhesus monkeys are hypersensitive to the negative feedback inhibition of LH secretion by follicular phase levels of E2 compared with dominant animals, suggesting that this hypersensitivity to E2 negative feedback inhibition of LH likely accounts for the increased frequency of anovulation or short luteal phase ovulations known to be a characteristic of female macaques exposed to psychogenic stressors (Adams et al., 1985; Kaplan et al., 1984; Walker et al., 1983; Williams et al., 2007; Xiao et al., 2002). Furthermore, the present results emphasize that genetic polymorphisms should be evaluated in the analysis of stress-induced reproductive deficits, as this hypersensitivity to E2 inhibition was accelerated in subordinate females with the s-variant 5HTT genotype. Importantly, by the end of treatment, social status differences in serum LH were no longer evident, suggesting the system was maximally suppressed at that time by this dose of E2. Nevertheless, these observations add support to pervious reports that individuals with this genotype are more vulnerable to the adverse consequences of social subordination (Jarrell et al., 2008) or other types of psychosocial stress (Barr et al., 2003; Bennett et al., 2002; Capitanio et al., 2008; Champoux et al., 2002; Suomi, 2003). This finding should be expanded to a larger population analysis to confirm whether 5HTT polymorphisms potentiate stress-induced reproductive compromise via enhanced E2 negative feedback inhibition.

The importance of E2 in differentiating LH secretion in dominant and subordinate females was underscored by the lack of social status differences in serum LH during the placebo condition. These observations contrast those from sheep in which psychosocial

stress can inhibit LH release in ovariectomized animals not replaced with E2 (Wagenmaker et al., 2008). Indeed, under the placebo conditions of the present study, females with the s-variant 5HTT genotype, regardless of dominance rank, had higher concentrations of serum LH. Assuming individuals with the short promoter length allele of the 5HTT gene may have reduced 5HT activity (Lesch et al., 1997), these higher levels of serum LH during the non-estradiol treatment periods in s-variant females could be explained by reduced serotonergic inhibition, as 5HT inhibits LH release during hypoestrogenic conditions (Dow et al., 1994; Vitale and Chiocchio, 1993). The biological significance of these genotype differences in the open loop secretion of LH is unclear at this time.

While overall levels of LH were reduced significantly more in subordinate compared to dominant females during E2 treatments, the differences were most pronounced during the initial days of E2 treatment, as serum LH was maximally suppressed in all females by day 7 of E2 treatment. This hypersensitivity to E2 negative feedback inhibition is similar to that observed during the initial stages of puberty (Rapisarda et al., 1983; Wilson, 1995; Wilson et al., 2004b), during lactational infertility (Wilson, 1993), and in the regulation of LH secretion in seasonally breeding animals (Karsch et al., 1993; Wilson et al., 1987). The inhibitory action of E2 is likely mediated through a number of neurochemical changes acting on hypothalamic gonadotropin releasing hormone (GnRH) release as well as directly affecting pituitary gonadotropin secretion (Goodman et al., 2002; Messinis, 2006; Moenter et al., 2003). However, the broader question is how this inhibitory action of E2 on LH is enhanced or exacerbated in socially subordinate females. Because social subordination in macaques is considered a

potent psychosocial stressor (Capitanio et al., 1998; Kaplan and Manuck, 1999; Sapolsky, 2005), characterized by a dysregulated LHPA axis (Chapter 2,(Abbott et al., 2003; Shively et al., 1997b; Wilson et al., 2008)), the most parsimonious explanation is that the increased suppression of LH in subordinate females is due to stress hormones acting synergistically with E2. However, because body weights were significantly lower in subordinate compared with dominant females one cannot rule out the possibility that metabolic signals also synergize with stress hormones (Williams et al., 2007) to enhance E2 negative feedback inhibition of LH.

A large body of data supports the hypothesis that CRH and glucocorticoids inhibit LH release. Central administration of CRH inhibits the secretion of LH in female rats and monkeys (Breen et al., 2008; Olster and Ferin, 1987; Petraglia et al., 1987) and the continuous expression of CRH from the central nucleus of the amygdala disrupts estrous cycles in female rats (Keen-Rhinehart et al., 2009), as CRH may act directly on GnRH cell bodies that project to the median eminence (Dudas and Merchenthaler, 2002; MacLusky et al., 1988). Furthermore, activation of pituitary glucocorticoid receptors (GRs) inhibits LH pulsatility (Breen et al., 2008; Breen et al., 2007; Saketos et al., 1993). There are few studies showing the importance of E2 on the stress-induced inhibition of LH. Estradiol potentiates the inhibition of LH by CRH in rats (Tsukamura et al., 1994) as well as the hypoglycemic stress-induced inhibition of LH (Adam and Findlay, 1998; Li et al., 2003; Tsukahara et al., 1999) at doses that have little effect on LH in non-stressed animals (Maeda et al., 1996). Furthermore, the reduction of LH pulse amplitude (Pierce et al., 2008b) and inhibition of GnRH release in ewes (Breen and Karsch, 2004; Oakley et al., 2008) by the pharmacological elevation of serum cortisol is dependent on E2.

Despite these observations that E2 enables or facilitates stress-induced inhibition of LH secretion, the mechanisms responsible for this synergistic action are not understood. We observed a small but significant decrease in serum cortisol during the E2 treatment phase when LH levels were most suppressed in all females. Thus, it would seem that the synergistic effect of E2 and glucocorticoids is explained by E2 up regulating GRs or post receptor signaling (Burgess and Handa, 1992; Sheng et al., 2003).

The hypersensitivity to E2 negative feedback inhibition of LH in subordinate females was associated with significantly higher rates of harassment from more dominant animals, a characteristic of macaque social status relations (Bernstein et al., 1974; Kaplan, 1987). Importantly, the current data indicate that subordinate, s-variant females received significantly more harassment from their more dominant s-variant cage mates and these behaviors were associated with an earlier maximum suppression of LH during the E2 treatment phase. The higher rates of aggression and affiliation exhibited by dominant s-variant females suggest these animals initiate more social interactions. Dysfunction of the 5HT system is linked to increased incidences of aggressive behavior (Kantak et al., 1984; Sanchez and Hyttel, 1994; Vergnes et al., 1988) as 5HT usually acts to inhibit aggression (Summers et al., 2005) and limit impulsivity (Hollander and Rosen, 2000). Previous studies indicate 5HT tone is lower in individuals with an s-variant genotype (Hoffman et al., 2007; Manuck et al., 2004; Reist et al., 2001) and reduced central 5HT activity is associated with increased impulsivity and aggression (Higley and Linnoila, 1997; Hollander and Rosen, 2000; Manuck et al., 2003; Westergaard et al., 2003; Westergaard et al., 1999) as well as hostility in humans (Reist et al., 2003; Williams et al., 2003). While s-variant females may be more predisposed to be aggressive

given the correct social circumstances (i.e., high dominance status), simply engaging in more social interactions could increase the likelihood that an agonistic episode will occur.

Although we observed a significant effect of social status on serum LH during the E2 treatment condition, the present study failed to show a difference in morning cortisol between dominant and subordinate females. It is important to note that only the results of a dexamethasone suppression test have previously described the dysregulated LHPA axis characteristic of socially subordinate macaques (Kaplan et al., 1984; Shively et al., 1997a; Wilson et al., 2008; Wilson et al., 2005). Thus, the analysis of a single morning sample for cortisol may provide limited power to detect these group differences and consequently be a poor surrogate measure of social status differences in LHPA regulation. Indeed, the lack of a relationship between morning cortisol and social status has been seen in other studies of female macaques (Gust et al., 1993; Stavisky et al., 2001). Furthermore, previous studies showing how exposure to a range of stressors induces ovulatory defects do not report serum cortisol (Centeno et al., 2007a; Williams et al., 2007) but rather report these reproductive deficits are associated with greater CRH expression in hypothalamic-limbic regions (Centeno et al., 2007b). Other parameters of the LHPA status, including provocative tests and more frequent sampling, may provide better biomarkers of psychosocial stress exposure that link stress exposure to reproductive compromise.

As described in *Materials and methods*, we chose a 10 mg/kg dose of the CRH type 1 receptor antagonist CP154526, an analog of the widely used antalarmin, to test the hypothesis that antagonism of CRH type 1 receptors would normalize LHPA dysregulation in subordinates and thereby improve LH secretion. However, we found the

daily administration of this drug for five consecutive days increased serum cortisol in all females and potentiated E2 negative feedback suppression of circulating LH in all females but the subordinate s-variant females. Because serum LH in these subordinate females was the lowest during the E2-only treatment condition compared to other females, it is possible that LH could not be further reduced by the effect of the drug. Previous studies show that the effects of CP154526 on corticosteroid release and behavior are inconsistent, as both low and high doses of the antagonist have elicited increases and decreases in stress hormone release and anxiety-like behavior in both rodents (Arborelius et al., 2000; Bornstein et al., 1998) and non-human primates (Ayala et al., 2004; French et al., 2007; Habib et al., 2000). Specifically in adult male monkeys a low dose of antalarmin (<3.2 mg/kg) antagonizes CRH-induced increases in ACTH, while a high dose of 10 mg/kg stimulates both cortisol and ACTH in adult male monkeys (Broadbear et al., 2004). While the mechanism of this effect is unknown, our results corroborate these data. Finally, the administration of CP154526 alone decreased serum LH but the effect was marginally significant ($p = 0.057$). While the effect of the drug in combination with E2 could be attributed to the small but statistically significant elevation in serum cortisol, one cannot rule out the possibility that the drug itself is acting on hypothalamic or pituitary targets to directly reduce LH secretion.

In summary, these data suggest that the reproductive compromise characteristic of subordinate females is due to a hypersensitivity to the negative feedback inhibition of E2 on circulating LH. Furthermore, serum LH was maximally suppressed sooner during E2 treatment in those subordinates with the s-variant 5HTT genotype, suggesting they may have increased susceptibility to this hypersensitivity. This reproductive phenotype

exhibited by subordinate females was associated with greater harassment by more dominant animals. The importance of cortisol as a signal on the hypothalamic – pituitary axis to inhibit LH must be further evaluated, as the paradoxical increase in serum cortisol by the CRH receptor antagonist potentiated this E2 inhibitory activity in all females, obscuring social status differences in LH secretion as the treatment progressed. Although our data indicate that the 5HTT genotype should be considered when evaluating risk factors for stress-induce infertility in women, these results must be considered preliminary as much larger cohorts of either animals or women are needed to establish a link between this gene variant and susceptibility to stress induced anovulation.
[End of text from (Michopoulos et al., 2009a)]

5.4 Effects of social subordination and 5HTT genotype on estradiol positive feedback of luteinizing hormone

5.4.1 Introduction

The findings from our first study (5.3) indicate that enhanced E2 negative feedback inhibition of LH accounts for the stress-induced disruption of reproduction in subordinate females (Michopoulos et al., 2009a). These data indicate that hypersensitivity to E2 negative feedback inhibition of LH in subordinate females would also result in low levels of both E2 and LH, thus precluding subordinate females from mounting an LH surge necessary for ovulation. However, other forms of reproductive dysfunction, including luteal phase deficiency (Walker et al., 1983) and estrogen deficiency (Adams et al., 1985), can also manifest themselves in subordinate macaques

(Kaplan and Manuck, 2008). This increased incidence of luteal phase deficiency in a subset of subordinate females suggests that the ability of E2 to induce an LH surge could be disrupted in these females. Thus, the next study was designed to test the hypothesis that subordinate females would be hyposensitive to E2 positive feedback of LH and that any such effect would be exacerbated by the presence of the short promoter length allele in the 5HTT gene. We hypothesized that subordinate females would show decreased sensitivity to E2 positive feedback of LH and that the presence of the s-variant allele would exacerbate the consequences of subordination on E2 positive feedback of LH. In this study, we used ovariectomized females receiving low dose E2 replacement therapy that subsequently received a single injection of E2 to mimic the increase in E2 that occurs at ovulation in gonadally intact cycling females (Walker et al., 1984).

5.4.2 Materials and methods

Subjects. Subjects were 45 ovariectomized adult female rhesus monkeys that were housed in one of eight small social groups at the YNPRC Field Station (n = 6 per group) comprising of 5 females and one adult male. The social status by genotype groupings were: 9 dominant l/l; 8 dominant s-variant; 14 subordinate l/l, and 14 subordinate s-variant. Groups had been formed for 48-54 months prior to the start of the present study and were ovariectomized 42 months prior to the formation of these groups. This study was performed in the months of January through and April, during the late breeding season.

Hormone replacement. All subjects received E2 replacement via implantation of E2 filled Silastic capsules subcutaneously as described previously (Michopoulos and

Wilson, 2011; Mook et al., 2005). Achieved serum levels of E2 following capsule implantation averaged 90.2 ± 28 pg/ml, comparable to the mid-folliclar phase levels of E2 (Walker et al., 1984). Ten days following capsule implantation, females were injected with a single subcutaneous 6.4 μg/kg dose of E2 immediately following a baseline serum sampling at 0800 hr to raise serum E2 levels to levels similar to what is noted during the preovulatory surge (~400 pg/ml) in intact adult rhesus females (Walker et al., 1984). Additional serum samples were obtained at five, ten, 24 and 48 hours following E2 administration to assess E2 levels and changes in LH due to E2 administration.

Outcome measures. Aggressive and submissive behavior for each female was recorded for 30 minutes to verify social status ranks. Inter-observer reliability exceeded 90%. Collected serum samples were assayed for both E2 and LH.

Statistical analyses. Data were summarized as mean ± SEM. A response of serum LH to injection of E2 was defined as a 20% increase in LH compared to baseline levels before administration of E2 injection. Eleven females did not meet this criterion and were dropped from further analyses. A fisher's exact test indicated that the proportion of non-responders was not different among dominant and subordinate females ($p = 0.284$). The main effects of status (dominant vs. subordinate), 5HTT genotype (l/l vs. s-variant), and hours on following E2 injection (0, 5, 10, 24, 48 hr), as well as their interactions on hormonal data from the 34 responding females were analyzed with analysis of variance for repeated measures. Two-tailed tests with a $p \leq 0.05$ were considered significant.

5.4.3 Results

Social Status based on agonistic behavior. Figure 5.5 shows rates of aggression received and submissive behavior emitted for monkeys at each social dominance rank position. Amount of aggression received ($F_{4, 35} = 8.17$, $p<0.001$) and submission emitted by females ($F_{4, 35} = 4.11$, $p=0.008$) varied significantly by social rank. Categorizing females ranked 1 and 2 as dominant and those ranked 3 through 5 as subordinate results in a significant main effect of status submissive behaviors emitted ($F_{1,38} = 9.73$, $p=0.003$) and increased levels of aggression received ($F_{1, 38} = 11.9$, $p=0.001$).

Serum E2. Serum E2 levels following E2 replacement did not vary by social status, 5HTT genotype or their interaction ($p > 0.05$). E2 levels were significantly affected by time following E2 administration (Figure 5.6A; $F_{4, 120} = 52.5$, $p < 0.001$). Serum E2 levels were significantly elevated from baseline (47.8 ± 4.237 pg/ml) at 5 (270 ± 29 pg/ml) hrs following E2 injection (Figure 5.6A). By 10, 24 and 48 hr following E2 administration, E2 levels were back down to baseline levels (Figure 5.6A). This main effect of time did not interact with status, 5HTT genotype or a status by 5HTT genotype interaction ($p > 0.05$).

Serum LH. Levels of LH were not significantly affected by social status ($F_{1,30} = 1.61$, $p = 0.214$), by 5HTT genotype ($F_{1,30} = 0.535$, $p = 0.470$) or by a status – 5HTT interaction ($F_{1,30} = 0.304$, $p = 0.585$). LH levels in responders were significantly affected by hours following E2 administration ($F_{1,30} = 3.93$, $p = 0.005$), as LH levels in all females at 24 hours following E2 injection were higher than LH levels at baseline, 5, 10, and 48 hrs following E2 administration (Figure 5.6B). This effect of time was not altered by social status, 5HTT genotype or a status by 5HTT genotype interaction ($p > 0.05$).

5.4.4 Discussion

In this study, we set out to determine whether subordinate females would be hyposensitive to E2 positive feedback of LH and that such an effect would be exacerbated by the presence of the short promoter length allele in the 5HTT gene. Our reasoning for this was that a blunting of E2's ability to induce an LH surge could account for luteal phase deficiencies observed in subordinate females (Kaplan and Manuck, 2008; Walker et al., 1983).

E2 replacement was achieved to mimic the patterns observed in E2 during the follicular and preovulatory phase of the ovarian cycle. E2 capsule administration raised E2 levels to basal follicular levels found in intact rhesus females (~90 pg/ml) (Walker et al., 1984; Wallen et al., 1984). Additionally, subsequent E2 injection elevated E2 levels to those comparable to the peak in E2 that precedes ovulation and the LH surge (~400 pg/ml) (Walker et al., 1984; Wallen et al., 1984). Thus, our hormonal manipulation of E2 in this study was successful in mimicking breeding season physiological levels of E2 during the follicular and preovulatory period. However, this dosing regimen did not increase LH in all females. In females responding with a 20% increase in serum LH levels following E2 injection, there were no affects of social status and 5HTT genotype on LH levels in response to E2 administration. Taken together with our findings from Study 5.3, these data indicate that hypersensitivity to E2 negative feedback inhibition of LH in subordinate females would preclude levels of both E2 and LH to rise and block the induction of a LH surge necessary for ovulation.

The inability to induce a rise in LH in all females following E2 injection could be due to limitations of the approach used in this study. The dose of E2 (6.4 μg/kg, SC)

used to mimic the rise of E2 during the preovulatory phase of the ovarian cycle was too low and did not result in a prolonged elevation of E2 as seen preovulatory phase of the ovarian cycle (Walker et al., 1984; Wallen et al., 1984). E2 levels during the follicular phase increase steadily (Walker et al., 1984; Wallen et al., 1984) as opposed to a sudden rise in E2 like we achieved in this experiment. To definitively assess whether social subordination affects E2 positive feedback of LH, this study should be repeated in the heart of the breeding season, and an estradiol benzoate injection should be used instead of E2 to induce a rise in E2 that is maintained for a longer period of time.

5.5 Does increased caloric intake diminish E2 negative feedback in subordinate females?

5.5.1 Introduction

While subordination in macaques is a potent psychogenic stressor that clearly increases LHPA drive and disrupts the HPG axis, it also elicits adverse metabolic changes in subordinate animals when animals are fed a typical low caloric laboratory diet. Subordinate females weigh significantly less than dominant animals, have significantly lower estimates of total body fat and are hypoleptinemic, results that directly complement the finding that subordinates have reduced levels of fat mass in comparison with dominant animals (Michopoulos et al., 2012a). While this reduction in body weight could be linked to the anorectic actions of LHPA axis activity and decreased food intake in subordinate monkeys, it could also be due to increased activity levels in subordinates. However, the possibility that energy regulation is globally disrupted in subordinate females still remains.

To test the hypothesis that metabolic differences between dominant and subordinate females due to alterations in feeding behavior are responsible for the reproductive alteration in E2 negative feedback of LH seen in subordinates (determined in Study 5.3), we provided animals with a diet choice, where animals had access to a high fat, high sugar diet (HFSD) and a standard low calorie diet (LCD). Based on our preliminary findings (Arce et al., 2010), we tested the hypothesis that increased caloric intake by subordinates during the dietary choice condition would normalize the E2 negative feedback of LH associated with subordinate status. The current study was done following the second week of the study described in Chapter 4, where animals had access to both a LCD and HFSD for a full two weeks prior to E2 replacement.

5.5.2 Materials and methods

Animals. Previously ovariectomized adult female rhesus monkeys (n = 39) living in indoor-outdoor enclosures at the Yerkes National Primate Research Center (YNPRC) Field Station (in groups of 4 and 5 females and 1 male) were used as subjects. The social status by genotype groupings were: 7 dominant l/l; 8 dominant s-variant; 12 subordinate l/l, and 12 subordinate s-variant. Groups had been formed for 35 months prior to the start of the present study and were ovariectomized 21 months prior to the formation of these groups. This study was performed in the months of October through and December, during the breeding season. All animals had access to the diets *ad libitum* via previously validated automated feeders that allow for continuous quantification of individual caloric intake (Arce et al., 2010; Wilson et al., 2008).

Design. Females were studied for two three-week diet condition phases separated by a three-week washout period. Each phase consisted of either a dietary choice between a low calorie monkey chow (LCD; 3.45 kcal/g, Purina 5038) and a high fat and sugar diet (HFSD; 3.73 kcal/g Purina Typical American Diet #5038) or access to only the LCD (no choice condition). The order of diet condition presentation was counterbalanced so that half the females received the choice condition first and the other half the no choice condition first. The caloric composition of the LCD was 12% fat, 18% protein, and 4.14% sugar carbohydrate and 65.9% fiber carbohydrate. The calories of the HFSD were distributed as 36% fat, 18% protein, 16.4% sugar carbohydrate and 29.6% fiber-starch carbohydrate. Body weights and blood samples for the analysis of serum leptin, insulin, and glucose were taken at the beginning and end of each phase of the study to assess changes due to diet availability.

Hormone replacement and sampling. During the third week of each diet phase, E2 replacement was achieved by implanting E2 filled Silastic capsules subcutaneously as described previously (Michopoulos and Wilson, 2011; Mook et al., 2005) to assess E2 negative feedback inhibition of LH. Capsules were implanted one day prior to the initiation of data collection and removed immediately following the end of the six day phase. Serum samples were collected at baseline (prior to E2 replacement) and at 24, 48, and hr following E2 replacement for the analyses of circulating E2 and LH levels.

Statistical analysis. Data were summarized as mean ± SEM. LH data were log transformed. The main effects of status (dominant vs. subordinate), 5HTT genotype (l/l vs. s-variant), diet condition (no choice vs. choice) and days following E2 implantation (0, 1, 2, 3, 4, 5), as well as their interactions on hormonal data were analyzed with

analysis of variance for repeated measures. Two-tailed tests with a $p \leq 0.05$ were considered significant.

5.5.3 Results

Social Status based on agonistic behavior. As shown in Figure 5.7, the amount of aggression received ($F_{4, 34} = 2.61$, p=0.05) and submission emitted by females ($F_{4, 34} = 3.39$, p=0.019) varied by social rank. Categorizing females ranked 1 and 2 as dominant and those ranked 3 through 5 as subordinate results in a significant main effect of status on submissive behaviors emitted ($F_{1, 37} = 7.35$, p=0.010) and increased levels of aggression received ($F_{1, 37} = 5.79$, p=0.021).

Serum E2. Serum E2 levels following E2 replacement did not vary by diet condition, social status, 5HTT genotype or their interaction ($p > 0.05$). E2 levels were significantly affected by time following E2 administration (Figure 5.8A; $F_{5, 175} = 5.74$, $p < 0.001$). Serum E2 levels were significantly elevated from baseline (11.2 ± 1.54 pg/ml) on days one (30.2 ± 1.64 pg/ml), two (28.8 ± 1.96 pg/ml), three (38.8 ± 8.8 pg/ml), four (51.1 ± 9.88 pg/ml), and five (29.1 ± 1.69 pg/ml) following E2 capsule implantation. This main effect of time did not interact with diet condition, status, 5HTT genotype or interactions with these factors ($p > 0.05$).

Serum LH. LH levels following E2 administration varied by day following E2 administration ($F_{5, 185} = 40.3$, $p<0.001$) in a manner that interacted significantly with social status ($F_{5, 175} = 28.4$, $p<0.001$) but not dependent on dietary condition ($F_{5, 175} = 1.83$, p=0.109) or the interaction between social status and diet condition ($F_{5, 175} = 0.684$, p=0.636). While baseline levels of LH were similar in dominant and subordinate

females, initial negative feedback was greater in dominant animals ($p=0.043$; Figure 5.8B). LH levels on Day 3 exhibited increased LH levels that were greater in dominant females compared to subordinates ($p=0.020$; Figure 5.8B). Day 4 LH levels were decreased in comparison to day 3 levels with greater suppression in subordinates compared to dominant females ($p=0.038$; Figure 5.8B). Diet condition and 5HTT genotype did not affect overall levels of LH in females ($p > 0.05$).

5.5.4 Discussion

In this study, we hypothesized that increased caloric intake during a dietary choice condition where a HFSD was available would normalize the E2 negative feedback of LH associated with subordinate status that was described in section 5.3 of this chapter. This study was done as a component of the feeding study presented in Chapter 4. However, while presentation of a HFSD during the choice diet condition for two weeks resulted in significantly higher calorie intake in subordinate females and not dominant females (as reported and discussed in Chapter 4), social status differences in E2 negative feedback inhibition of LH was unaffected by this increased caloric intake in subordinate females.

One possible explanation for the lack of an effect of increased caloric intake in subordinate females on E2 negative feedback inhibition of LH is that increased caloric intake during the three-week period failed to substantially increase metabolic signals in subordinate females important for neuroendocrine regulation of reproduction. Under conditions where only a LCD diet is available, subordinate females weigh less than dominant animals, are hypoleptinemic, and have reduced levels of fat mass in comparison

with dominant animals (Michopoulos et al., 2012a). Leptin signaling from adipose tissue normally acts to stimulate GnRH in monkeys (Finn et al., 1998). However, while leptin levels were increased in subordinate females in association with increased caloric intake during the dietary choice (Table 4.2), a significant change in body weight was not observed. These data indicate that acute changes in metabolic signals that are observed in subordinate females in response to short term increases in energy balance are not sufficient to affect E2 negative feedback on LH. It is possible that a longer exposure to the rich dietary environment would have diminished E2 negative feedback in subordinates. However, central administration of leptin does not restore ovarian activity in calorically restricted monkeys (Lujan et al., 2006) indicating that changes in leptin signaling are not sufficient for restoring reproductive function.

Despite the current data not supporting our primary hypothesis, the patterns of LH secretion following E2 administration that achieved follicular levels of E2 in all females differed in some but not all ways described in Study 5.3. While baseline levels of LH were similar in both dominant and subordinate females, initial E2 negative feedback of LH was greater in dominant females compared to subordinate females. While these data initially seem contrary to those presented in Study 5.3, a careful examination of the differences between the methods used in the two studies shows that both studies indicate that E2 negative feedback inhibition of LH is enhanced in subordinate females. In Study 5.3, E2 replacement occurred on Day 0, and follow up blood samples collected on days 3-7. In the current Study 5.4, E2 replacement occurred on Day 0, and follow up blood samples collected on days 1-5. Thus, days 1-4 of Study 5.4 are equivalent to days 3-7 in

Study 5.3 and in both studies on these days subordinate females show greater inhibition of LH levels in the presence of E2.

Finally it is important to note that in the current study, there was no effect of 5HTT genotype on E2 negative feedback inhibition of LH. The lack of an effect of 5HTT most likely is a result of Study 5.6 being underpowered to assess the effects of social status, 5HTT genotype, and diet condition because of counterbalancing the order of the diets. To more definitively make a conclusion about the role of 5HTT genotype on these endpoints, the ideal study described above should not only be conducted so that animals are exposed to HFSD over a period of 6-12 months, but also involve a greater number of subjects so that the study is appropriately powered to detect potential effects of 5HTT genotype.

5.6 Conclusions

In this chapter, we have presented a series of experiments that describe the reproductive phenotype of subordinate females in comparison to dominant animals. We show that the psychosocial stress of social subordination results in a hypersensitivity to the negative feedback inhibition of LH secretion by follicular phase levels of E2 that is exacerbated by the s-variant 5HTT genotype. This study also supported the hypothesis that cortisol is an important signal on the hypothalamic – pituitary axis to inhibit LH, as the paradoxical increase in serum cortisol by the CRH receptor antagonist potentiated the E2 inhibitory activity in all females, obscuring social status differences in LH secretion as the treatment progressed. We conclude that the increased frequency of anovulation or short luteal phase ovulations characteristic of female macaques exposed to psychogenic

stressors (Adams et al., 1985; Kaplan et al., 1984; Walker et al., 1983; Williams et al., 2007; Xiao et al., 2002) are due to hypersensitivity to E2 negative feedback inhibition of LH that also results in low levels of both E2 and LH that would preclude the ability of subordinate animals to mount an LH surge necessary for ovulation and not the ability to mount an LH surge in the presence of adequate E2 levels. Finally, we show that a two-week exposure to a dietary condition that results in increased caloric intake in subordinates but not dominant females does not diminish the enhanced inhibitory actions of E2 on LH levels observed in subordinate females. We hypothesize that a longer period of increased caloric intake is necessary to induce biologically relevant changes in metabolism that might then mitigate the effects of psychosocial stress exposure on reproductive function in subordinate females.

Table 5.1. Rates (mean ± SEM) of aggressive and submissive behavior directed towards others illustrated by individual dominance ranks and by social status categories for females with an l/l and s-variant 5HTT genotype. The main effect of social status (dominant vs. subordinate) and 5HTT genotype were significant ($p < 0.05$) for both behaviors as indicated by different superscripts for each classification. *[From (Michopoulos et al., 2009a)]*

	Aggression Towards Others		Submissive Towards Others	
Rank/Status	l/l[a]	s-variant[b]	l/l[a]	s-variant[b]
1	3.20 ± .41	4.90 ± .72	0	0
2	2.35 ± 1.1	7.78 ± 2.2	0.19 ± 0.13	0.10 ± 0.35
3	2.30 ±1.8	5.23 ± 1.9	0.44 ± 0.51	1.39 ± .83
4	1.43 ± 1.2	5.70 ± 1.9	0.63 ± .83	3.30 ± 1.1
5	0	0	1.98 ± 1.1	6.80 ± 0.83
Dominant[1]	2.78 ± 1.2	5.34 ± 1.3	0.11 ± 0.91	1.62 ± 0.99
Subordinate[2]	0.94 ± 1.8	3.65 ± 1.13	0.37 ± 1.01	4.32 ± 0.86

Table 5.2. Body weights (mean ± SEM) for dominant (Dom) and subordinate (Sub) at each genotype (l/l, s-variant) at the beginning and conclusion of each of the four treatment phases. Significant main effect of status ($p < 0.05$) is indicated by different superscripts for each classification. *[From (Michopoulos et al., 2009a)]*

	Control		E2		CRHA		E2 + CRHA	
	Start	End	Start	End	Start	End	Start	End
Dom, l/l	9.01 (0.44)	8.90 (0.46)	9.18 (0.48)	9.13 (0.48)	9.37 (0.47)	9.17 (0.46)	9.24 (0.45)	9.10 (0.44)
Dom, s-variant	7.76 (0.44)	7.70 (0.46)	7.93 (0.48)	7.76 (0.48)	7.74 (0.47)	7.66 (0.46)	7.83 (0.45)	7.56 (0.44)
Sub, l/l	7.11 (0.42)	6.98 (0.43)	7.27 (0.45)	7.13 (0.46)	7.46 (0.44)	7.28 (0.44)	7.26 (0.42)	7.09 (0.42)
Sub, s-variant	7.06 (0.36)	7.07 (0.37)	7.18 (0.39)	7.00 (0.39)	6.97 (0.39)	6.85 (0.38)	7.06 (0.37)	6.81 (0.36)
Dominant[1]	8.38 (0.31)	8.30 (0.32)	8.56 (0.34)	8.44 (0.34)	8.55 (0.33)	8.41 (0.33)	8.54 (0.32)	8.33 (0.31)
Subordinate[2]	7.08 (0.28)	7.03 (0.29)	7.23 (0.30)	7.06 (0.30)	7.21 (0.29)	7.07 (0.29)	7.16 (0.28)	6.95 (0.28)
l/l	8.06 (0.30)	7.94 (0.31)	8.22 (0.33)	8.13 (0.33)	8.41 (0.32)	8.22 (0.32)	8.25 (0.31)	8.10 (0.30)
s-variant	7.41 (0.29)	7.39 (0.30)	7.56 (0.31)	7.38 (0.31)	7.35 (0.30)	7.26 (0.30)	7.44 (0.29)	7.18 (0.29)

Figure 5.1. Mean ± SEM hourly rates for aggressive behavior received from other animals as a function of an animal's dominance rank (#1 - #5) and 5HTT genotype (left panel) and as a function of social status (dominant, ranks 1 and 2 vs. subordinate (ranks 3 – 5) and genotype (right panel). Different letters among the four groups during each treatment condition indicate groups differed significantly ($p < 0.05$). *[From (Michopoulos et al., 2009a)]*

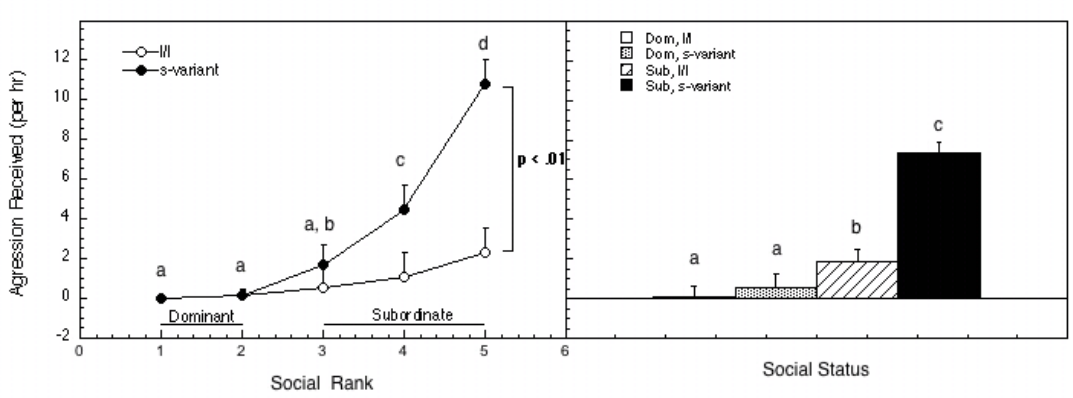


Figure 5.2. Mean ± SEM serum concentrations of morning cortisol during each of the four treatment conditions in dominant females with an l/l (open bar) and s-variant 5HTT genotype (gray bar) and subordinate females with an l/l (stippled bar) and s-variant 5HTT genotype (solid bar). *[From (Michopoulos et al., 2009a)]*

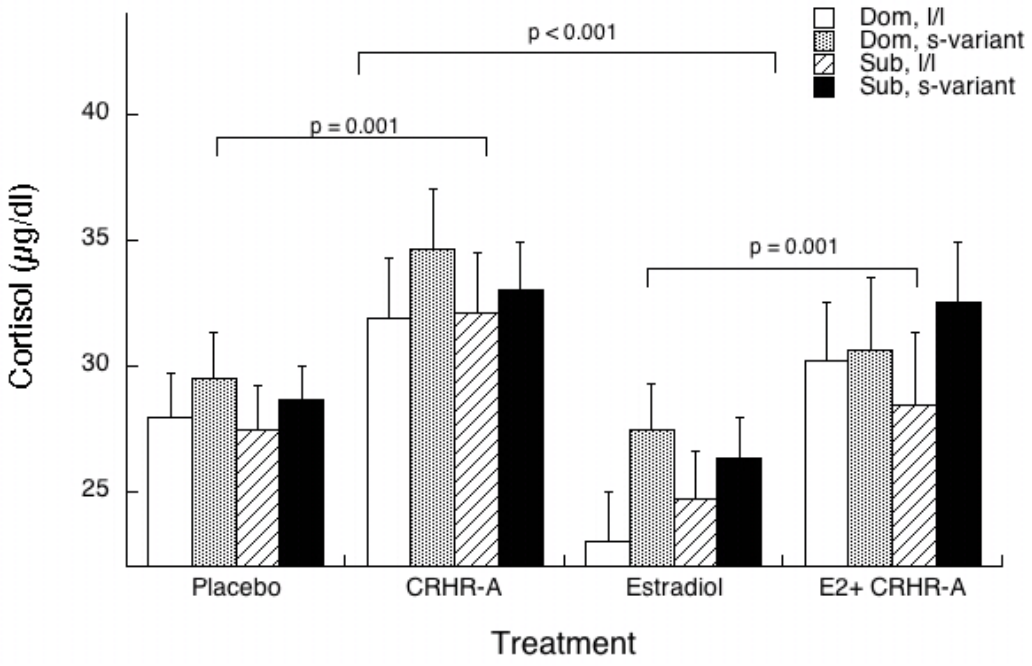


Figure 5.3. Mean ± SEM serum concentration of LH during the Control (no treatment) condition and CRHA condition (panel B) treatment conditions as a function of social status (dominant versus subordinate) and 5HTT genotype (l/l and s-variant). Asterisks indicate significant differences between groups and treatments ($p < 0.05$). *[From (Michopoulos et al., 2009a)]*

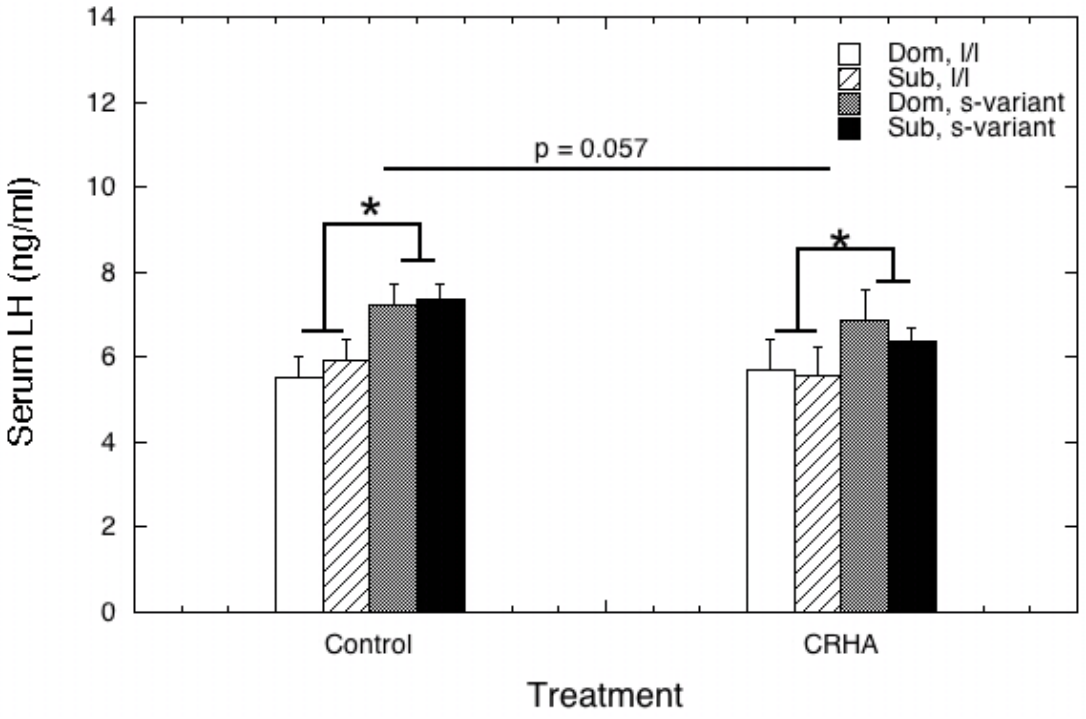


Figure 5.4. Mean ± SEM serum concentrations of morning LH on days 4 through 7 of treatment with E2 only (left panel) and E2 plus CRHA (right panel) as a function of social status (dominant versus subordinate) and 5HTT genotype (l/l and s-variant). Asterisks indicate significant differences between groups ($p < 0.05$). *[From (Michopoulos et al., 2009a)]*

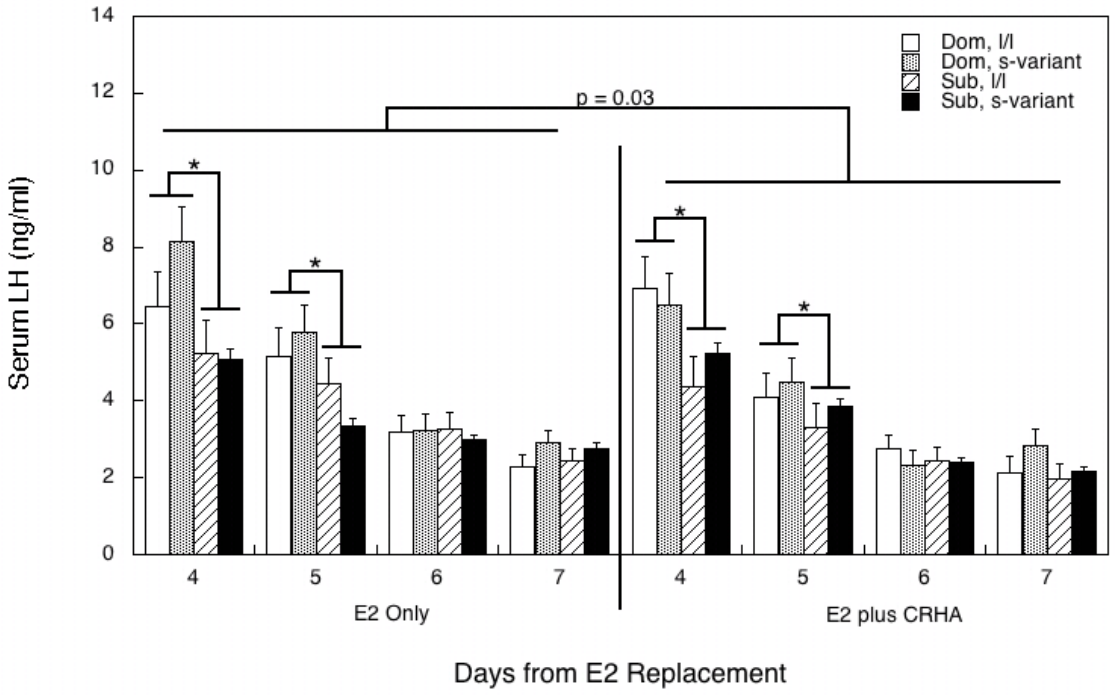


Figure 5.5. Mean ± SEM rates (per 30 min) of aggressive behavior received and submission behavior emitted by females at each social dominance rank. Rates of aggression received ($p = 0.001$) and submission emitted ($p = 0.003$) were higher in animals categorized as subordinate females (ranks 3 – 5) compared with those categorized as dominant (ranks 1 and 2).

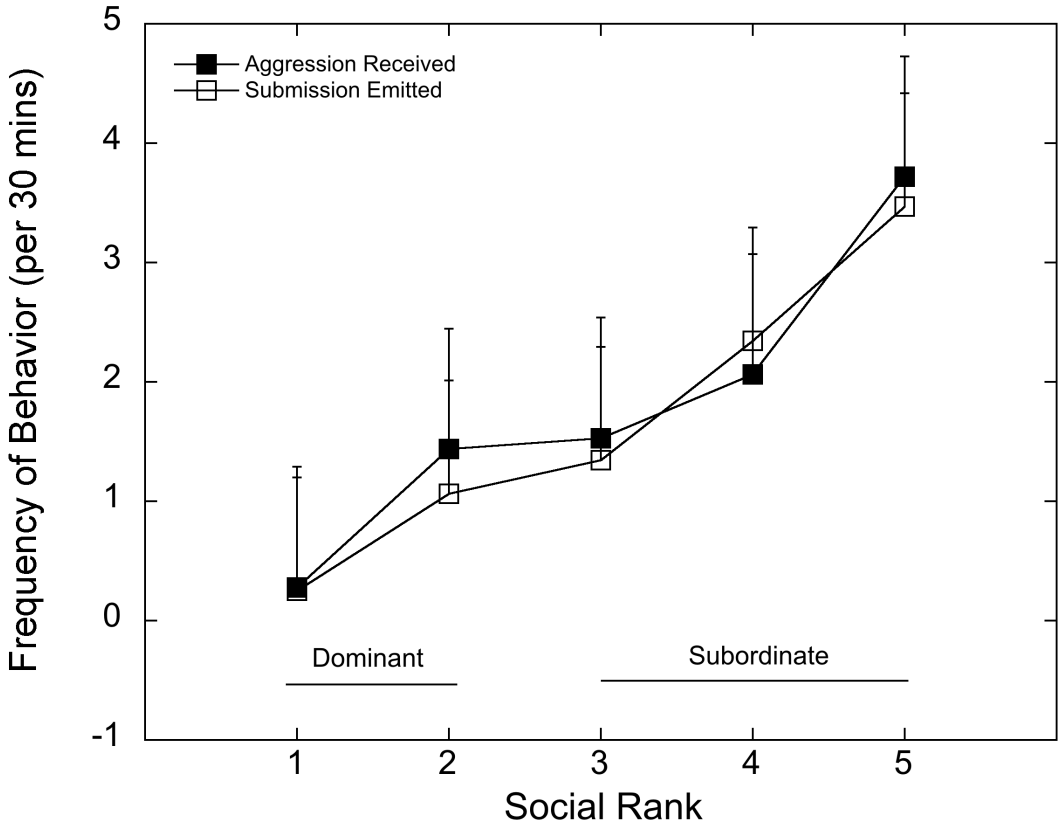


Figure 5.6. Mean ± SEM levels of (A) estradiol and (B) LH broken down by social status and 5HTT genotype. The asterisk in (A) denotes a significant increase in serum estradiol from baseline levels in all females, regardless of social status and 5HTT genotype. The asterisk in (B) signifies a higher overall level of LH in all females at 24 hrs following E2 injection compared to baseline and all time points following estradiol administration.

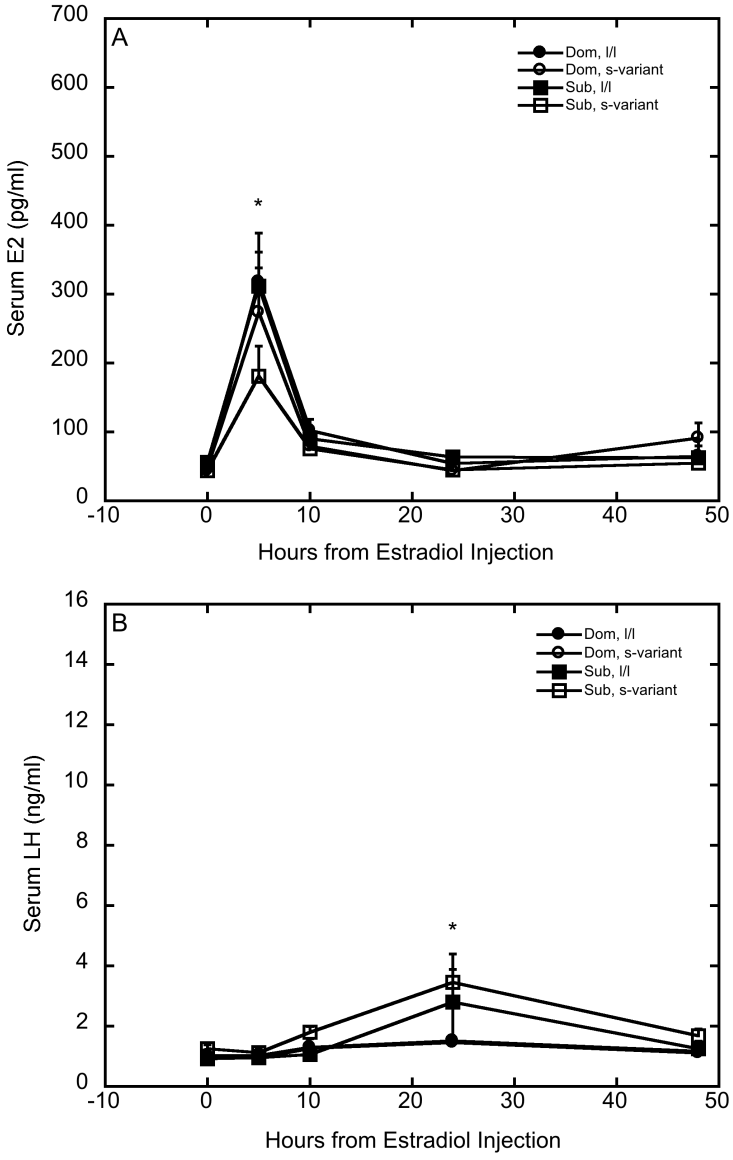


Figure 5.7. Mean ± SEM rates (per 30 min) of aggressive behavior received and submission behavior emitted by females at each social dominance rank. Rates of aggression received ($p = 0.021$) and submission emitted ($p = 0.010$) were higher in animals categorized as subordinate females (ranks 3 – 5) compared with those categorized as dominant (ranks 1 and 2).

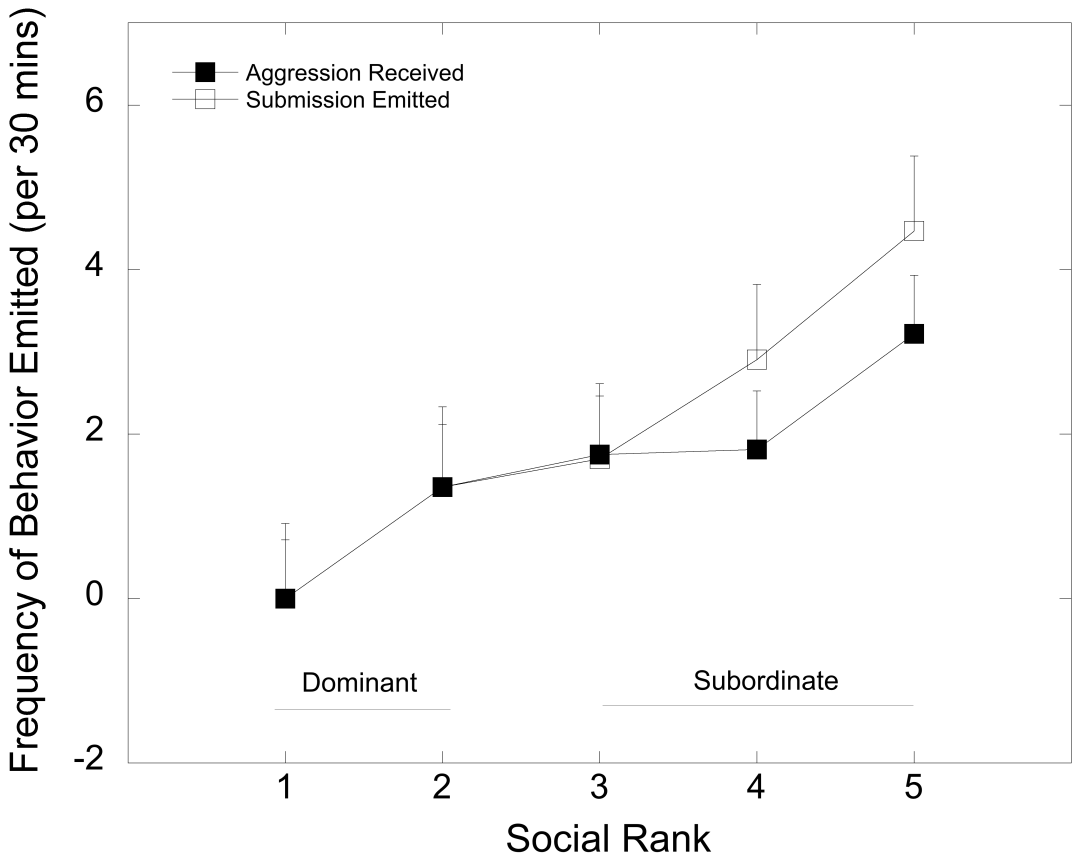
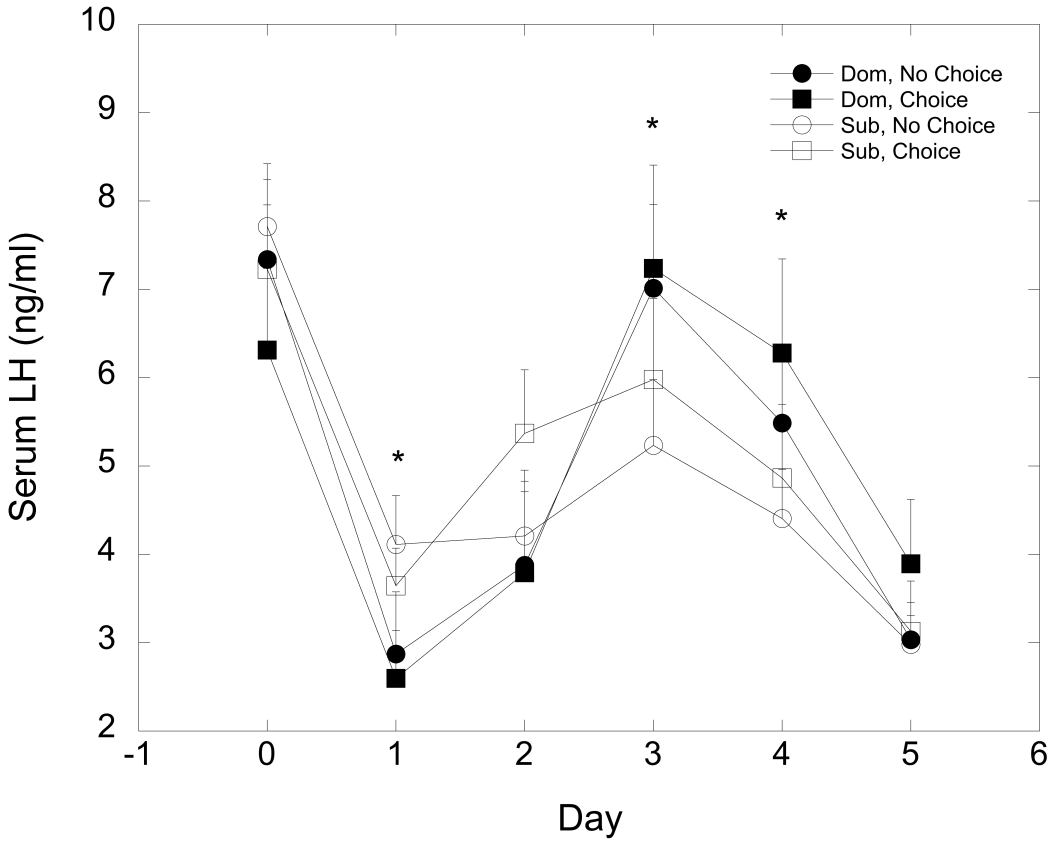


Figure 5.8. Mean ± SEM levels LH broken down by social status and diet condition. The asterisks denote status differences in LH levels on days one, three and four following E2 replacement ($p < 0.05$).



CHAPTER SIX:

TWO DISTINT PHENOTYPES EMERGE FROM SOCIAL SUBORDINATION IN FEMALE RHESUS MONKEYS

6.1 Summary

The overall goal of this dissertation was to define whether social subordination in female rhesus macaques results in consistent differences in LHPA regulation and whether these differences produce phenotypes that are known to be stress dependent in females. An exploratory emphasis in this dissertation was to assess how the 5HTT polymorphism influences individual vulnerability to adverse consequences of psychosocial stressor exposure. To address our overall aim, we evaluated the effects of social status and 5HTT genotype on four modalities, including stress axis function (Chapter 2), social and anxiety-like behavior in the presence of the ovarian hormone estradiol (Chapter 3), food intake and metabolic profile under specific dietary conditions (Chapter 4), and reproductive physiology (Chapter 5).

In Chapter 2, we show that subordinate females, when compared to dominant females, exhibit blunted morning cortisol secretion, diminished glucocorticoid negative feedback, and decreased adrenal cortisol response to an ACTH challenge. These results indicate that the ability to mount and limit glucocorticoid release is significantly reduced by psychosocial stress in female rhesus macaques, indicating a dysregulation of the LHPA axis that renders the axis hyporesponsive, similar to what is observed in several human psychopathologies, including post traumatic stress disorder (Chrousos and Gold, 1992; Raison and Miller, 2003). These data also show that dexamethasone suppressed serum cortisol and ACTH administration increased serum cortisol progressively more with increasing social ranking (Figure 2.6). Importantly, these data validate the conventional use of categorizing dominant females as animals ranked 1 and 2 and subordinates as those ranked 3 – 5 in the literature (Kaplan, 2008; Kaplan et al., 1995;

Kaplan et al., 1984; Kaplan et al., 2010; Kaplan et al., 1982; Kaplan et al., 2002; Paiardini et al., 2009; Shively and Kaplan, 1984; Shively, 1998b; Shively and Clarkson, 1994; Shively et al., 1997a; Shively et al., 1997b) and in the subsequent studies of this dissertation.

Coincident with LHPA dysregulation, we demonstrate in Chapter 4 that socially subordinate females engage in emotional feeding when a diet choice is offered between a high fat, sugar diet (HFSD) and a standard low calorie diet (LCD). While all females preferred the HFSD, only subordinates increased their overall caloric intake by a third during the choice phase. Interestingly, this increased calorie intake was maintained in subordinate monkeys even after withdrawal of the HFSD when only the "healthy" LCD was available. The degree of HFSD, but not the LCD, intake during the choice condition was predicted by the degree of reduced glucocorticoid negative feedback as assessed with a dexamethasone suppression test. The cortisol response to an acute stressor also significantly predicted subsequent intake of a HFSD in all females. These data are consistent with those showing intake of calorically dense foods increases LHPA activity in rodents (Kamara et al., 1998; Tannenbaum et al., 1997) and in humans (Pasquali et al., 2002), and indicate how emotional feeding might be a self-sustaining behavior by inducing and not diminishing the activity of the LHPA axis. Greater energy resources due to HFSD intake release more cortisol to mobilize these increased energy resources while concurrently changing food salience and increasing caloric intake (Warne, 2009).

In Chapters 2 and 5, we addressed whether psychosocial stressor exposure in the form of subordination and 5HTT genotype influenced social and self-directed behaviors as well as reproductive physiology in the presence of estradiol (E2). E2 increased

affiliative behaviors in all animals, but with dominant females receiving even more of these prosocial behaviors. S-variant females, regardless of social status, were more aggressive towards more subordinate cage mates and these behaviors too were increased by E2. Subordinate s-variant females were most often involved in agonistic behavior, emitted less affiliative behavior, and were less responsive to the anxiolytic action of E2. Additionally, we observed that subordinate females were hypersensitive to E2 negative feedback inhibition of luteinizing hormone (LH), an effect exacerbated by the presence of the s-variant 5HTT polymorphism. These results show that the short allele of the 5HTT gene can synergize with psychosocial stress exposure to affect the behavioral and reproductive efficacy of E2.

Taken together, the data described in this dissertation support the notion that social subordination in female rhesus monkeys results in two distinct phenotypes by disrupting behavior and physiology in subordinate individuals, and supports the notion that chronic stressor exposure is a causal and sustaining factor in a number of adverse health outcomes (Juster et al., 2010; McEwen, 2008). However, while each of the studies was independently conducted assessing the effects of subordination on questions within specific physiological and behavioral domains in the same cohort of females, it remains uncertain how social subordination influences behavior and physiology overall in individual monkeys and whether the social dominance hierarchy results in distinct phenotypes that reflects the differential exposure to social stress on behavior and physiology in females. To synthesize these diverse data sets, we performed a discriminate analysis on variables collected to more completely evaluate the subordinate

phenotype and provide evidence that social subordination results in two distinct behavioral and physiological phenotypes.

[All text and figures from (Michopoulos et al., 2012a)]

6.2 Materiel and methods

Data were collected on 39 adult female rhesus monkeys that had been studied for 6 years over the course of this dissertation work. The intent of the study was to describe a broad range of phenotypical endpoints to determine what differentiates dominant from subordinate females. Most data were obtained prospectively as described below while selected previously published data from this same cohort of females were also included. Specifically, social status differences in serum concentrations of LH in response to low dose E2 negative feedback inhibition (Chapter 5, (Michopoulos et al., 2009a)) as well as status differences in serum oxytocin during estradiol replacement (Chapter 3, (Michopoulos et al., 2011b)) were included. In addition, the samples used for the oxytocin analyses were additionally analyzed for pituitary adenylate cyclase activating peptide (PACAP), a retroactive molecule thought to be stress sensitive in females (Ressler et al., 2011).

Prospective data were obtained on females in the absence of hormone replacement with an average washout period 4.85 ± 0.13 weeks with a range of 4-6 weeks since their last estradiol replacement. Data were collected in two different phases, separated by a minimum of 3 weeks of no assessments. The first phase was six weeks in duration. During this period, animals had continual access to the typical low fat, high fiber nonhuman primate diet (Test Diets, #5038, Richmond IN) comprised of 3.50

kcal/gram with 18% of calories derived from protein, 12% from fat, 60% from fiber, and 10% from sugar. The purpose of this phase was to obtain a range of phenotypes, including neurochemical, LHPA responsivity, metabolic, and anthropometric data, while females consumed a standard laboratory diet. During the first week, animals received a dexamethasone (Dex) suppression test to determine glucocorticoid negative feedback (Wilson et al., 2008). On day 1, a baseline serum sample was obtained at 1100 hr. At 1730 hr, each female received an injection of Dex (0.25 mg/kg, IM) and samples were obtained at 1100 hr the following morning for the assay of cortisol. Absolute values of cortisol following Dex as well as the change in cortisol from the post Dex to the baseline sample were used in the analysis. During week 3, females received an ACTH stimulation test (Shively, 1998b). At 0800 hr, a serum sample was obtained followed by an injection of Dex (0.5 mg/kg, IM) to suppress endogenous ACTH. Four hours later, another serum sample was obtained followed by an injection of Cortrosyn (10 ng/kg, IV). Subsequent samples were obtained at +15 and +30 min. The area under the curve in serum cortisol was used for the analysis.

In addition to the assessment of LHPA regulation, a single blood sample was obtained at the beginning of week 4 and week 5 for the analysis of metabolic hormones, including plasma ghrelin and serum fructosamine (as an index of circulating glucose over a 2 to 3 week period), insulin, leptin, and adiponectin. Following the sample collection at week 5, whole body scans using a dual x-ray absorptiometry (DEXA; Norland Eclipse) were performed to obtain total lean, fat, and bone mass. Collars were then placed on each animal (Primate Products) and an Actical Accelerometer (MiniMiter, Bend OR) was attached to record activity bouts. The devises were programed to sum activity every 30

sec. Collars were removed 7 days later and hourly activity outs were calculated throughout a 24-hr day averaged across the 7-day period. Animals were anesthetized for the removal of the collars. At this time, a sample of cerebrospinal fluid (CSF) was obtained from the cisterna magna for the measurement of the serotonin (5HT) metabolite 5 hydroxyindoleamine (5HIAA) as well as dopamine (DA) and its metabolites homovanillic acid (HVA) and 3, 4-dihydroxyphenylacetic acid (DOPAC). Finally, body weights were obtained on each animal every week. In addition to these metabolic and physiological measures, behavioral data using an established ethogram were collected for 30 min twice weekly during the 6 weeks of the initial phase as described previously (Jarrell et al., 2008).

Finally, the intent of the second phase of the study was to characterize total caloric intake when females were presented with a choice between the typical low fat, high fiber monkey diet described above (3.54 kcal/gram) and a diet with additional fat and sugar (3.73 kcal/gram), having 18% of calories derived from protein, 36% from fat, 31% from fiber, and 15% from sugar (Test Diets, "Typical American Diet"). The rationale for including these data is that based on previous reports from our laboratory that subordinate females consume significantly more calories when a calorically dense diet is made available (Arce et al., 2010). Diets were presented to the animals through previously validated automated feeders that quantifies food intake continually for individual animals housed in a social setting (Arce et al., 2010; Wilson et al., 2008). Each group had access two feeders one containing the low caloric diet and one containing the high caloric diet for two weeks. A complete description of feeding behavior under these different dietary environments is contained in Chapter 4.

Statistical analyses. MANOVA and Discriminant Analysis (DA) procedures were chosen to analyze these data for which variables maximize the differences between dominant and subordinate subjects, because these procedures (a) protect against Type I error inflation problems caused by running multiple separate ANOVAs for each separate variable and (b) help reveal potential combinations of variables that optimally differentiate the groups dominant and subordinate females (Field, 2009; Tabachnik and Fidell, 2007). All data were complete and no significant outliers were noted. Right skewed distributions were noted for some of the variables; however, the MANOVA and DA procedures used are robust to deviations from normality (Tabachnik and Fidell, 2007). The 33 variables were included into either a Behavioral, Metabolic, or Neuroendocrine domain as listed in Table 6.1. Prior to analysis, Z-scores were created for each variable to eliminate any variable comparison biases due to scale differences or offset from zero. The first step in analysis was to review the pairwise correlations among all variables within each domain. Any variables that had high correlations ($r > 0.65$) were considered for possible removal because highly co-linear variables will cause instabilities within the matrix inversion procedures required by MANOVA and DA. Multicollinearity problems were also handled within MANOVA and DA procedures by removing any variables that failed the tolerance test from further analysis. An initial DA procedure was performed to see which variables had discriminant function "loadings" ≥ 0.3, as variables with higher loadings contribute more to group differentiation (Field, 2009; Tabachnik and Fidell, 2007). Only one discriminant function was possible for the data set because only two groups were considered (dominant versus subordinate). Any variables with loadings < 0.3 were removed from analysis for the final DA. This final variable list was

additionally run through the MANOVA procedure to calculate the extent to which each separate variable significantly differentiated between the 2 groups. The power for each discriminant analysis/MANOVA was calculated using PASS 2008 (NCSS, LLC; Kaysville, Utah) inputting the 2 group means for each of the variables retained in the final model as well as the pooled within groups covariance matrix. Additionally, t-tests, adjusting degrees of freedom for unequal variances, were performed on the resulting discriminant scores for each model comparing dominant and subordinate groups with accompanying effect sizes (Rosnow, 2003). Finally, for each discriminant function, receiver operating characteristic (ROC) curves and their associated areas under the curve (AUC) were calculated for each domain as a final comparison for each domain's classification accuracy.

6.3 Results

Social status based on agonistc behavior. Data are shown in Figure 6.1 to illustrate the well-accepted social rank differences in the mean frequency of aggression received and submissive behavior that supports our categorization of dominant and subordinate females. Females ranked 3 – 5 received significantly more aggression from higher-ranking group mates ($F_{4, 27} = 11.4$, $p < 0.001$). This harassment was associated with rank-dependent, higher rates of submissive behavior ($F_{4, 27} = 6.56$, $p < 0.001$). Importantly, rates of aggression directed towards others do not differ significantly between dominant and subordinate females ($p > 0.05$).

Behavioral Results. Table 6.2 presents the DA and MANOVA results for the variables considered in the Behavioral domain. No high correlations were noted and no

variables failed the tolerance test. However, only 4 of the 8 variables in this domain yielded loadings of 0.3 or higher: aggression received from others, submission directed towards others, frequency of affiliation directed towards others, and affiliation received from others which were retained in the final model. Activity levels, duration of time in affiliation, aggression directed towards others and anxiety-like behavior all had loadings < 0.3 and were excluded from the analysis. In the final DA model, aggression received and submission emitted loaded the highest onto the discriminant function followed by affiliative behaviors initiated and received. Only aggression received and submission showed significant between group effects within the MANOVA procedure. As can be seen in Figure 6.2, the dominant females had lower rates of aggression received from others, submission directed towards others, and affiliation received from others yet higher rates of affiliation directed towards others.

The final discriminant function explained approximately 45% (Canonical R^2) of the variability between predictors and Status groups. The final discriminant analysis model correctly classified 87.2% of the cases when using the leave-one-out cross-validation procedure. This MANOVA design achieved 99% power to test the Status factor given a Wilks' Lambda of 0.546 at a 5% significance level.

Metabolic Results. Table 6.3 presents the DA and MANOVA results for the variables considered in the Metabolic Domain. Weight was removed from analysis because it was highly correlated ($r > 0.65$) with several other measures including insulin, fat mass and bone mass. The next DA yielded 5 measures with loadings of 0.3 or greater: fat mass, average kcal consumed, bone mass, leptin, and adiponectin. Ghrelin, fructosamine, insulin, and lean mass all had loadings < 0.3 and were excluded from the

analysis. In the final DA model, fat mass, average kcal consumed during the choice diet phase and bone mass loaded highest onto the discriminant function, followed by leptin and adiponectin. All of these showed significant ($p<0.05$) between group effects within the MANOVA procedure. As can be seen in Figure 6.3, the dominant females exhibited significantly higher fat mass, bone mass, and leptin, and reduced adiponectin compared to subordinate females. Again, these data were obtained while animals consumed the standard low fat monkey diet. In contrast, when presented with a choice between this diet and a high caloric diet, total average kcal consumed was significantly higher for subordinate compared to dominant females (Figure 3).

The final discriminant function explained 40% (Canonical R^2) of the variance between the predictors and Status groups. The final DA model correctly classified 84.6% of the cases when using the leave-one-out cross-validation procedure. This MANOVA design achieved 96% power to test the Status factor given a Wilks' Lambda of 0.604 at a 5% significance level.

Neuroendocrine Results. Table 6.4 presents the DA and MANOVA results for the variables considered in the Neuroendocrine domain. No high correlations were noted and no variables failed the tolerance test. However, only 6 of the 12 variables in this domain yielded loadings of 0.3 or higher: serum LH in response to estradiol negative feedback, cortisol in response to an ACTH challenge, serum oxytocin, CSF concentrations of DOPAC, the degree of glucocorticoid negative feedback, and serum PACAP which were retained in the final model. CSF levels of DA, HIAA, and HVA as well as post Dex cortisol concentrations had loadings < 0.3 and were removed from further analysis. Serum LH in response to estradiol negative feedback, cortisol in

response to an ACTH challenge, serum oxytocin loaded the highest onto the discriminant function, followed by DOPAC, degree of glucocorticoid negative feedback, and PACAP. None of these last three had significant between group effects within the MANOVA procedure. As can be seen in Figure 6.4, the dominant females exhibited higher serum LH in response to estradiol negative feedback, cortisol in response to an ACTH challenge, serum oxytocin, CSF DOPAC and serum PACAP and a greater degree of glucocorticoid negative feedback than did the subordinate females.

The final discriminant function explained approximately 42% (Canonical R^2) of the variability between predictors and Status groups. The final discriminant analysis correctly classified 74.4% of the cases using the leave-one-out cross-validation procedure. This MANOVA design achieved 96% power to test the Status factor given a Wilks' Lambda of 0.578 at a 5% significance level.

Shown in Figure 6.5 are boxplots of the discriminant scores for dominant and subordinate females. As a comparison of the models fit for each of the 3 domains, t-tests and effect sizes were performed on the scores for each model comparing dominant and subordinate groups. The Behavioral domain yielded the largest effect size (ES) of d=-2.27 ($t_{31.16} = 6.34$, $p < 0.01$), followed by the Neuroendocrine domain model (ES d=2.05; $t_{37} = 4.68$, $p < 0.01$) and finally the Metabolic domain model (ES d=-1.62, $t_{20.87} = 4.93$, $p < 0.01$). Similar comparison of the 3 domains was also made using receiver operating characteristic curves (ROC; Figure 6.6) generated from the discriminant analysis scores (Figure 6.5) and associated areas under the curve (AUC) calculated where the positive state was the subordinate rank (Tables 2-4). The behavioral domain had the highest AUC (0.946) indicating that the discriminant function based on the behavioral domain achieved

the best classification accuracy, followed by the neuroendocrine domain (AUC = 0.894) and the metabolic domain (AUC = 0.878).

6.4 Discussion

The current discriminate analysis of a number of behavioral, metabolic/anthropometric, and neuroendocrine variables demonstrates that social status in female rhesus monkeys produces two distinct phenotypes and is consistent with the hypothesis that social subordination in female macaques is a potent and chronic psychosocial stressor (Sapolsky, 2005). This analysis of phenotypic social status differences in female rhesus monkeys compliments the well-established social status differences observed in behavior and physiology of cynomolgus macaques (Kaplan and Manuck, 2008; Shively and Willard, 2011) and marmoset monkeys (Saltzman et al., 2009).

6.4.1 Behavioral domain

As expected, the behavioral domain best discriminated social status in socially housed female rhesus monkeys based on ROC curve analysis. It is not surprising that rates of aggression received and submission emitted were the best predictors of social status, as these behaviors are used to determine social ranking in macaques (Bernstein, 1976; Bernstein and Gordon, 1974; Bernstein et al., 1974; Bernstein and Mason, 1970; Shively and Kaplan, 1984). The frequency of engaging in and receiving affiliative behavior from cage-mates also contributed to the classification of dominant and subordinate females, as dominant females most often initiate affiliation with subordinates

being the most typical target. The observation that dominant animals are most often the target of affiliative behaviors in groups devoid of males (Chapter 3, (Michopoulos et al., 2011b)) underscores the importance of the social context for determining the pattern of prosocial behaviors (Jacobs and Petit, 2011; Lehmann and Ross, 2011). In addition, subordination in female macaques has been associated with higher rates of anxiety (Wilson et al., 2008) or depressive-like behaviors (Shively et al., 1997b). However, in the present analysis measures of emotionality were excluded because of low loading scores. The emergence of anxiety-like behaviors in macaques may depend on a number of contextual situations such as competition for mates or other resources and because subordination delays access to those resources the situation may produce increases in these emotional behaviors. Furthermore, analyses of female cynomolgus monkeys indicate only a subset of subordinate females, with even fewer dominant females, exhibit depressive-like behavior (Shively and Willard, 2011). Nonetheless, the present analysis shows that within the context of the present social configuration the incidence of prosocial behaviors is influenced by social status.

6.4.2 Metabolic domain

In addition to distinct differences in social behavior, metabolic and anthropometric variables differentiated subordinate from dominant females. When animals had access to a standard laboratory monkey diet, body composition, such as fat and bone mass, as well as the peripheral metabolic markers leptin and adiponectin, best discriminate social status categories. The pattern of these markers in subordinate animals compared to dominant animals is consistent with the notion that exposure to chronic

stress and resulting LHPA dysregulation produces a subclinical state of negative energy balance (Arce et al., 2010; Belzung and Anderson, 1986; Gamaro et al., 2003; Tamashiro et al., 2004). Peripheral adiponectin levels are inversely related to adiposity (Spranger et al., 2003) and act to increase insulin sensitivity (Galic et al., 2010). However, despite adiponectin clearly differentiating dominant and subordinate females, the status differences in serum insulin did not load to contribute significantly to the DA. Because activity levels did not vary by social status, it is likely these differences are attributable to differences in ingestion of this low caloric diet (Michopoulos et al., 2009b), a notion consistent with a number of studies in laboratory rodents of stress-induced anorexia (Gamaro et al., 2003; Jochman et al., 2005; Marti et al., 1994; Smagin et al., 1999). However, when the dietary environment of the monkeys changed to include a choice of a high caloric diet more closely modeling what people experience, food intake clearly differentiated social status categorizations with subordinate females consuming more overall calories than dominant animals. This result is consistent with the idea that exposure to psychosocial stressors increases preference for and intake of highly palatable food in laboratory animals (Berridge, 1996; Dallman et al., 2005; Hagan et al., 2003; Tamashiro et al., 2006) and women (Adam and Epel, 2007; Epel et al., 2001). Because the monkeys only had access to the diets for two weeks, no significant change in adiposity markers were observed, despite the significantly higher caloric intake by subordinates (Chapter 4). Longer exposure to this rich dietary environment will determine how indices of adiposity increase in subordinates. Indeed, studies of female cynomolgus females fed a high fat, atherogenic diet for 32 months indicates more often subordinates develop more abdominal fat than do dominant monkeys (Shively et al.,

2009). However, because food intake was not quantified in the Shively et. al. 2009 study, it is not known whether this increased visceral fat is due to increased consumption of the diet and/or stress-induced redistribution of fat stores.

6.4.3 Neuroendocrine domain

A number of neuroendocrine endpoints contributed significantly to the DA. The hypersensitivity to estradiol negative feedback inhibition of LH secretion in subordinates (Chapter 5, (Michopoulos et al., 2009a)) is consistent with subordination-induced increase in the incidence of anovulation in macaques (Kaplan et al., 2010; Pope et al., 1986; Shively et al., 1997b) and marmoset monkeys (Abbott and Hearn, 1978; Saltzman et al., 2009) as well as psychosocial stress-induced infertility in ewes (Pierce et al., 2008a) and women (Homan et al., 2007; Nakamura et al., 2008; Wilson and Kopitzke, 2002). DOPAC reflects dopamine turnover and elevations in cortico–limbic regions are associated with enhanced executive control over emotion in both animals and people (Robbins and Arnsten, 2009). Thus, elevations in central DOPAC in dominant females may suggest enhanced emotional control. In addition, studies in macaques indicate the social subordination is associated with a hypodopaminergic condition (Grant et al., 1998; Kaplan et al., 2002; Morgan et al., 2002; Shively, 1998b) and increased vulnerability to psychostimulant self-administration (Morgan et al., 2002). Although CSF 5HIAA levels were lower in subordinates, this factor did not differentiate social status ranks, a finding consistent with previous studies (Kaplan et al., 2002). However, subordinate females do show reduced 5HT1A receptor binding in cortico-limbic regions (Shively, 1998b) and reduced expression of the 5HT precursor, tryptophan hydroxylase in the raphe (Shively et

al., 2003). Our previous report of higher serum oxytocin levels in dominant females (Chapter 3, (Michopoulos et al., 2011b)) contributed significantly to the DA as well. Oxytocin is implicated in increasing a vast array of social behaviors in both animals and human beings (Goodson and Thompson, 2010) and has distinct anxiolytic effects in animals (Steckler, 2010). Furthermore, increased oxytocin in dominant females is likely associated increased motivation to initiate affiliative behaviors described here. Finally, dysregulation of PACAP has recently been implicated in increased PTSD symptomology in women (Ressler et al., 2011), and reduced levels of this neurotropic peptide may render subordinate females more vulnerable to aversive events.

In the past, measures of LHPA axis function, including morning cortisol (Czoty et al., 2009; Gust et al., 1993; Stavisky et al., 2001) and overall diurnal cortisol levels (Arce et al., 2010; Collura et al., 2009), have been inconsistent in differentiating dominant from subordinate female macaques. Rather, socially subordinate female rhesus and cynomolgus macaques most often have increased adrenal size (Shively and Kaplan, 1984; Shively, 1998b) and decreased glucocorticoid negative feedback (Collura et al., 2009; Jarrell et al., 2008; Kaplan et al., 2010; Shively, 1998b; Shively et al., 1997b; Wilson et al., 2008) as clear indicators of LHPA dysregulation. The current analysis showed that the suppression of cortisol due to glucocorticoid negative feedback as assessed by a dexamethasone suppression test and the cortisol response to ACTH administration (Riddick et al., 2009; Shively, 1998b; Shively et al., 1997b) are the most potent and reliable LHPA axis predictors of social status in female rhesus macaques. Interestingly, dominant monkeys show significantly more cortisol release after ACTH administration than do subordinate monkeys. This has not been shown in previous studies in monkeys

(Riddick et al., 2009; Shively, 1998b; Shively et al., 1997b) and may be due to increased body fat in the dominant monkeys. It has been shown that increases in body fat can lead to a more robust glucocorticoid response in rats (Tannenbaum et al., 1997). In contrast to this pattern observed in macaques, subordinate female marmoset monkeys show suppressed serum cortisol (Saltzman et al., 1994), reflecting a blunted adrenal response to hypothalamic – pituitary activation (Saltzman et al., 2006). This hyporesponsiveness, albeit different than macaque females, nonetheless reflects a dysregulation of the LHPA axis and is a common feature of humans suffering from psychopathologies such as PTSD and depression (Berga et al., 1989; Epel et al., 2001; Juster et al., 2010; McEwen, 2008). Laboratory studies that experimentally manipulate group membership and reduce opportunities for social support can effectively exacerbate the consequences of stress-induced changes in emotionality (Jarrell et al., 2008; Kaplan et al., 1991; Morgan et al., 2000; Shively et al., 1997b). Thus, the extent of LHPA dysregulation and consequential stress-induced problems in macaque females are likely influenced by the amount of harassment subordinates receive and whether they have opportunities to engage in prosocial behaviors from family members or other conspecifics that may mitigate the stress (Abbott et al., 2003). Examining females of each specific subordinate rank in future studies may help to elucidate if there is, in fact, a graded LHPA response due to specific psychosocial stress level.

[End of text from (Michopoulos et al., 2012a)]

6.4.4 Limitations

Because these groups were formed by randomly taking females from the middle portion of the dominance hierarchy in their natal groups and combining them with

unfamiliar females (Jarrell et al., 2008), it is unlikely that female characteristics, independent of social status, account for the differences described in this dissertation. However, an unequivocal test of that hypothesis would require reforming groups to change the ranks of the females to ensure that, once the groups had stabilized, rank explained the distant phenotype (Shively et al., 1997b). Clearly, gene polymorphisms may also increase vulnerability to the consequences of social subordination (Chapter 1.4) as we have shown the short promoter length variant in the gene encoding the serotonin transporter (5HTT) exacerbates the suppression of LH secretion in subordinate females (Chapter 5, (Michopoulos et al., 2009a)) in response to estradiol administration. However, it is important to note that the data implicating the 5HTT genotype as a vulnerability marker for adverse health events following exposure to chronic psychosocial stressors should be considered preliminary as a larger number of subjects are necessary to establish a causal link between the 5HTT polymorphisms and vulnerability to stress-induced disruptions in behavior and physiology (Garcia-Closas and Lubin, 1999; Hwang et al., 1994). For this same reason, the lack of 5HTT genotype effects in Chapters 2 and 4 should be considered preliminary as the sample size in these studies and the number of factors being assessed might have underpowered the analyses.

Despite the statistically distinct differences that emerged, a limitation of the present studies is the rather narrow list of variables that were evaluated, as many systems are stress responsive. For example, immune function differs between dominant and subordinate females (Paiardini et al., 2009) and subordinate females are less behaviorally responsive to the activational effects of the ovarian hormone estradiol (Wallen, 2001). In addition, data from cynomolgus females show status differences in brain neurochemistry

based on PET studies (Grand et al., 2005; Grant et al., 1998). Other neuroimaging studies could show structural and functional differences between dominant and subordinate rhesus females. Indeed, a PET neuroimaging study assessing status differences in the GABAergic system using flumazenil is currently being analyzed and other studies assessing status differences in resting state activity and connectivity using MRI are underway.

Another critical point that could be considered both a strength and a weakness of the studies in this dissertation is the fact that many of the assessments were done in ovariectomized females in the absence of ovarian hormone replacement. While studying ovariectomized females receiving no hormone replacement could be considered a limitation because it provides a more similar context for postmenopausal women not receiving hormone replacement therapy than premenopausal cycling women, it is important to control for ovarian hormone status in females when studying physiology and behavior as both E2 and progesterone modulate the regulation of the systems studied as a part of this dissertation, including the LHPA axis (Wilson et al., 2005), social behavior (Chapter 3), feeding behavior and appetite regulation (Michopoulos et al., 2011a; Michopoulos and Wilson, 2011) and the reproductive axis (Chapter 5, (Michopoulos et al., 2009a)). Additional studies are necessary to determine how the phenotypes described in this dissertation are modified by the presence of E2 and progesterone, alone and in concert to provide a more similar context for postmenopausal women receiving hormone replacement therapy and premenopausal cycling women. Conducting these studies comparing the effects of ovarian hormones on behavior and physiology are critical in understanding the influence of these hormones on modulating individual vulnerability to

stress-induced disorders specifically in women who suffer from these disorders at a rate of two to one over men (Barry et al., 2008; Jones and Carney, 2006; Weissman and Olfson, 1995; Wurtman, 1993; Wurtman and Wurtman, 1995; Zellner et al., 2006; Zellner et al., 2007).

Furthermore, we recognize that the social grouping used in our studies is a "special case" with respect to normal macaque social organization. A similar analysis of adult females in large groups with multiple males and offspring may yield a somewhat different phenotypic pattern due to mitigating effects of social support from family members or other conspecifics (Abbott et al., 2003; Ozbay et al., 2008). In addition, it is important to note that use of other nonhuman primate models may yield a different phenotypic profile between dominant and subordinate females. For example, as described above, subordinate female marmosets have a blunted adrenal cortisol response (Saltzman et al., 2006) and subordinate status is not typically associated with more harassment from more dominant females (Abbott et al., 1998). Nonetheless, the approach used in the present study shows a continual exposure to the social stress of subordination in female rhesus monkeys, an experience that has species-specific ethological validity, has lasting effects on a number of phenotypes.

6.4.5 Possible mechanism underlying stress-induced changes in behavior and physiology

The etiology underlying the co-morbid disruptions in reproductive function, behavioral sensitivity to estradiol, and food intake and metabolism due to social subordination remains unclear. Central to these behavioral and physiological disruptions is the dysregulation of the LHPA axis described in Chapter 2, an observation that

parallels findings in humans with depression that exhibit diminished glucocorticoid negative feedback (Coryell et al., 2008; Jokinen et al., 2008). Studies in rodents show that altered glucocorticoid negative feedback due to exposure to chronic stressors changes the expression of genes critical for the regulation of the LHPA axis. Chronic stress exposure in rodents increases CRH expression in the CeA and the BNST (Albeck et al., 1997; Shepard et al., 2000) and CRH in the PVN has been shown to be upregulated by chronic stress states in rodents (Ma et al., 1999) and in humans with depression (Raadsheer et al., 1994). Concurrent with the upregulation in CRH expression within the CeA is a downregulation or upregulation of CRHR1 in the PVN, depending on the stressor employed (Bonaz and Rivest, 1998; Keen-Rhinehart et al., 2009).

It is important to note that most of the studies assessing neuroanatomical and neurochemical changes in gene expression due to chronic stressor exposure have been done in rodents and not in non-human primates. Anatomical differences in expression of CRH receptors, as well as glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) between rodents and macaques complicate the interpretation of how exposure to chronic stressors alters behavior and physiology. CRHR1 in the macaque brain generally overlap with areas in the rat brain and pituitary, while CRHR2 expression is found throughout the macaque brain, including limbic regions of the PFC (Sanchez et al., 1999). Additionally, in rodents GRs are expressed highly in the hippocampus (McEwen, 1997), whereas in macaques, GR mRNA is weakly detected in the hippocampus and abundant in the pituitary, PVN, and cortex (Sanchez et al., 2000). Taken together, these data indicate that the hypothalamus, pituitary and cortex might be more important neuroanatomical sites for the regulation of the LHPA axis in monkeys than the

hippocampus, or that mineralocorticoid receptors that are expressed in the macaque hippocampus mediate the hippocampal effects of glucocorticoids in macaques (Sanchez et al., 2000).

The involvement of the hypothalamus in the regulation of the LHPA axis is critical for the interaction of the LHPA axis with other neuroendocrine systems due to the neuroanatomical overlap of these systems at the level of the hypothalamus. The LHPA axis interacts with the reproductive axis by modulating GnRH pulsatility via both cortisol and CRH (Breen et al., 2005; Breen et al., 2008; Oakley et al., 2008, 2009; Olster and Ferin, 1987; Petraglia et al., 1987). The LHPA axis also interacts with neuroendocrine and metabolic systems modulating food intake via connectivity between the arcuate nucleus and PVN of the hypothalamus (Ricardo and Koh, 1978). Disruptions of food intake due to heightened activity of the LHPA axis that is characteristic of chronic stress conditions in humans and rodents are dependent upon diet type and availability, as well as the CRH-urocortin system (Barry et al., 2008; Dallman et al., 2002; Heiskanen et al., 2006; Richard et al., 2002). Urocortins promote negative energy balance by increasing energy expenditure and attenuating food intake by interacting with CRHR2 in the arcuate nucleus of the hypothalamus and the PVN (Fekete and Zorrilla, 2007; Lewis et al., 2001). Finally, the hypothalamic component of the LHPA axis in the PVN is also a critical center for the control of prosocial behaviors and is responsive to ovarian hormones, including estradiol (Pfaff et al., 2000; Rissman et al., 1997a), and oxytocin (Ross and Young, 2009).

While the hypothalamus is uniquely positioned to integrate signals from multiple neuroendocrine systems, it is the activity of higher order structures, including the

hippocampus, amygdala, prefrontal cortex (PFC), orbitofrontal cortex (OFC) that regulate the activity of the LHPA axis at the level of the hypothalamus (Herman et al., 2003). Temporal regions including the amygdala and hippocampus are connected to the PFC and OFC via the uncinate fasciculus and cingulum bundle (Zhang et al., 2011). The OFC inhibits the CeA via the uncinate fasciculus, thus disinhibiting amydalar output to hypothalamic structures (Barbas, 2007). Not only does exposure to chronic stressors in rodents, monkeys and humans result in alterations of neurotransmitter and neuropeptide systems, as discussed in Chapters 2, 4 and 5, but continuous stressor exposure induces morphological changes in limbic structures as well as the fiber tracts between them that are implicated in the etiology of disturbances in mood and cognition as seen in psychiatric disorders (McEwen et al., 2012; McLaughlin et al., 2009; Zhang et al., 2011). Cognitive dysfunction linked to PTSD and major depression is associated with decreased hippocampal volume (Bremner et al., 2000; Brown et al., 1999a; Karl et al., 2006; Vasic et al., 2008). The PFC is critical in executive processing (Dalley et al., 2004; Ragozzino, 2007) and in concert with the amygdala is involved with defining emotionally salient stimuli (Cahill and McGaugh, 1998; LeDoux, 2000). Additionally, the PFC is critical for responding to stressful stimuli by engaging in adaptive behavior (Cerqueira et al., 2008). Chronic restraint stress in rodents induces morphological changes within the PFC and the amygdala (McEwen et al., 2012; McLaughlin et al., 2009) that are associated with attention impairments (Liston et al., 2006). Humans with depression show decreased PFC volume (Bremner et al., 2002; Konarski et al., 2008) and increased amygdalar volume (Lange and Irle, 2004), though this result is not consistent in the literature (McLaughlin et al., 2009). These data taken together suggest that changes in neuronal

structure and circuitry observed in individuals suffering from similar psychopathologies are important in the etiology of their disorders. Importantly, a sexual dimorphism in the effects of chronic stress on morphological alterations in these LHPA regulatory centers corroborates the overall sex difference in the prevalence of stress-induced disorders in women (McLaughlin et al., 2009).

6.4.6 Future directions

Currently it is not known whether social subordination results in volumetric differences in the size of the hippocampus, amygdala and PFC in female macaques. Neuroimaging studies using magnetic resonance imaging (MRI) are underway to assess whether the size of these structures are indeed different between dominant and subordinate females, paralleling what is seen in human psychopathologies. In parallel with structural MRIs, connectivity between limbic areas implicated in regulating emotionality and the LHPA axis is being assessed via digital tensor imagine (DTI) as well as resting-state MRI. Using positron emission tomography (PET) techniques in concert with MRI studies to determine how social subordination alters brain neurochemistry within specific loci will become very important for determining the mechanism by which subordinate results in the behavioral and physiological phenotypes described in this dissertation.

Another important tool to be used in the future to define the etiology of subordination-induced adverse health effects is that of epigenetics, or the study of how the environment alters gene expression without altering the primary structure of DNA. Indeed, changes in gene expression underlying long-lasting changes in physiology and

behavior due to social subordination are likely due to epigenetic alterations in DNA methylation and histone modification (Tung et al., 2012), which are both implicated in mediating the accessibility of a specific segment of DNA to the transcriptional machinery necessary of its expression (Kadonaga, 1998; Razin, 1998). The seminal work on epigenetic modification of the genome was done looking at the environmental effects of postnatal neglect on the development of physiology and behavior in rats (Meaney, 2001). These studies showed that LHPA axis reactivity was lower in rats that were raised by mothers that engaged in licking and grooming (LG) behavior often as compared to rats that were raised by mothers that engaged in LG behavior less frequently (Meaney et al., 1989). Neurochemically, these differences in maternal behavior lead to higher expression of the GR within the hippocampus is associated with greater sensitivity to glucocorticoid negative feedback (Weaver et al., 2004). Cross-fostering mothers did not account for this difference, suggesting that environment of the specific dam and not the genetics of the birth mother influenced the expression pattern of hippocampal GR (Weaver et al., 2004).

Though studies looking at direct effects of environment on gene expression are harder to come by in non-human primates and humans, there is ample literature associating adverse environments with increased incidence of maladaptive behavior and altered physiology, leading to psychopathology and altered emotionality. Maternal abuse and neglect is endogenous to macaque species and maltreated offspring show abnormal social behavior as early as six months of age (Maestripieri et al., 1997; McCormack et al., 2006). Stress axis physiology is also heightened in maltreated offspring, as central levels of CRH are elevated and diurnal cortisol is flattened (Maestripieri et al., 2005; Sanchez et

al., 2005). In humans, abuse early on in life is linked to increased vulnerability to both physiological impairments (Danese et al., 2007; Felitti et al., 1998) and psychopathology (Kessler et al., 1994; MacMillan et al., 2001; Mullen et al., 1996), including depression (Brown et al., 1999b), addiction (Cohen and Densen-Gerber, 1982), and personality disorder (Johnson et al., 1999; Rogosch and Cicchetti, 2004). These examples in monkeys and in humans show that adverse environments have long-lasting effects on behavior and physiology similar to social subordination in macaques and offspring of low LG mothers in rodents, suggesting that similar epigenetic mechanisms might be involved in the etiology of these adverse health consequences.

6.5 Conclusion

In summary, the data presented in this dissertation show that a number of markers in subordinate females, including alterations in LHPA axis regulation, match those observed in humans who are under chronic stress and heavy allostatic load (Berga et al., 1989; Epel et al., 2001; Juster et al., 2010; McEwen, 2008), including changes that emerge with low socioeconomic status (SES) (Marmot, 2003; Miller et al., 2009). However, studies of social status in macaques is more than a model of SES, as subordinate status reflects a condition of unresolved and unpredictable recurring stressor exposure that individuals of any SES may experience. Results assembled in this dissertation suggest that social subordination in rhesus monkey females represents a unique translational model of psychosocial stress and a valuable means to investigate any number of adverse health effects of exposure to chronic psychosocial stressors that occur in females.

Table 6.1. List of Variables in Each Measurement Domain: Metabolic, Behavioral and Neuroendocrine. [*From (Michopoulos et al., 2012a)*]

Phenotype	**Dominant (n = 16)**		**Subordinate (n = 23)**	
Behavioral	**Mean**	**sem**	**Mean**	**sem**
Activity	6458.41	768.79	6253.27	485.00
Frequency of affiliation initiated (per hr)	3.28	0.55	2.04	0.34
Duration of time in affiliation (per hr)	14.10	2.02	14.95	2.96
Frequency of affiliation received from others (per hr)	1.76	0.32	2.71	0.35
Frequency aggression directed towards others (per hr)	2.10	0.56	1.32	0.56
Frequency of aggression received from others (per hr)	0.13	0.06	2.46	0.53
Frequency of submission directed towards others (per hr)	0.39	0.20	2.29	0.46
Frequency of anxiety-like behavior (per hr)	7.00	1.12	6.14	0.58
Metabolic				
Adiponectin (ng/ml serum)	15423.82	1928.09	20857.48	1522.13
Average kcals from a choice diet	130.77	13.65	184.55	14.90
Bone mass (g)	309.51	11.71	274.27	8.55
Fat mass (g)	2206.45	374.26	876.83	268.96
Fructosamine (mg/dl serum)	190.56	3.68	183.87	2.25
Ghrelin (pg/ml plasma)	11.75	2.64	13.64	2.12
Insulin (µU/ml serum)	60.42	19.55	45.58	15.96
Lean mass (g)	6363.50	146.49	6245.87	88.24
Leptin (ng/ml serum)	30.73	5.70	16.39	2.91
Weight (kg)	8.93	0.41	7.34	0.29
Neuroendocrine				
PACAP (pM serum)	126.73	7.15	112.89	5.22
DOPAC (ng/ml CSF)	5.71	0.63	4.11	0.56
DA (ng/ml CSF)	3.67	0.55	3.36	0.36
HIAA (ng/ml CSF)	90.14	10.34	80.56	7.80
HVA (ng/ml CSF)	501.11	40.07	461.34	26.48
Oxytocin(pg/ml serum)	216.52	24.07	160.09	12.65
Cortisol response to ACTH (plasma AUC)	79.00	16.59	37.01	9.29
Degree of glucocorticoid negative feedback (change in cortisol (µg/dl serum by Dex compared to control)	-23.91	2.73	-19.43	1.07
Post Dex serum cortisol (µg/dl serum)	3.95	0.87	4.10	0.65
LH (ng/ml serum) response to estradiol negative feedback	6.49	0.63	4.49	0.32

Table 6.2. Behavioral domain: DA and MANOVA Results. [*From (Michopoulos et al., 2012a)*]

Behavioral Predictor Variables (Z-scores used in analysis)	**Discriminant Function Loadings**	**MANOVA: Between-Groups Effects F-statistic (df1=1,df2=37); (p-value)**
Aggression received from others	0.654	13.163 (0.001)
Submission directed towards others	0.594	10.859 (0.002)
Affiliation directed towards others	- 0.365	4.099 (0.050)
Affiliation received from others	0.343	3.622 (0.065)
Discriminant Analysis Results		
Canonical R^2	0.454	
Eigenvalue	0.833	
Wilks' Lambda	0.546	
Chi-square,df (p-value)	21.209,4 (<0.001)	
Cross-Validation %	87.2% cases correctly classified (cross-validation using leave-one-out "jackknife" method)	
Area Under ROC Curve	AUC = 0.946, SE = 0.034, p < 0.001, 95% CI [0.878, 1.000]	
Variables Removed From Analysis		
Activity levels	Loading < 0.3	
Duration of time in affiliation	Loading < 0.3	
Aggression directed towards others	Loading < 0.3	
Anxiety-like behaviors	Loading < 0.3	

Table 6.3. Metabolic Domain – DA and MANOVA Results. [*From (Michopoulos et al., 2012a)*]

Metabolic Predictor Variables (Z-scores used in analysis)	**Discriminant Function Loadings**	**MANOVA: Between-Groups Effects F-statistic (df1=1,df2=37); (p-value)**
Fat Mass	- 0.601	8.790 (0.005)
Average kcal consumed	0.514	6.432 (0.016)
Bone Mass	- 0.505	6.201 (0.017)
Leptin	- 0.494	5.939 (0.020)
Adiponectin	0.453	4.993 (0.032)
Discriminant Analysis Results		
Canonical R^2	0.397	
Eigenvalue	0.657	
Wilks' Lambda	0.604	
Chi-square,df (p-value)	17.420,5 (0.004)	
Cross-Validation %	84.6% cases correctly classified (cross-validation using leave-one-out/"jackknife" method)	
Area Under ROC Curve	AUC = 0.878, SE = 0.076, p < 0.001, 95% CI [0.729, 1.000]	
Variables Removed From Analysis		
Ghrelin	Loading < 0.3	
Fructosamine	Loading < 0.3	
Insulin	Loading < 0.3	
Lean Mass	Loading < 0.3	
Weight	Highly correlated with Insulin (r=0.655), Fat Mass (r=0.951) and Bone Mass (r=0.698)	

Table 6.4. Neuroendocrine domain: DA and MANOVA Results. [*From (Michopoulos et al., 2012a)*]

Neuroendocrine Predictor Variables (Z-scores used in analysis)	**Discriminant Function Loadings**	**MANOVA: Between-Groups Effects F-statistic (df1=1,df2=37); (p-value)**
Serum LH in response to estradiol negative feedback	0.592	9.459 (0.004)
Serum cortisol response to ACTH challenge	0.456	5.607 (0.023)
Serum oxytocin	0.433	5.051 (0.031)
CSF DOPAC	0.361	3.518 (0.069)
Degree of glucocorticoid negative feedback	- 0.331	2.956 (0.094)
Serum PACAP	0.309	2.566 (0.118)
Discriminant Analysis Results		
Canonical R^2	0.421	
Eigenvalue	0.729	
Wilks' Lambda	0.578	
Chi-square,df (p-value)	18.612,6 (0.005)	
Cross-Validation %	74.4% cases correctly classified (cross-validation using leave-one-out "jackknife" method)	
Area Under ROC Curve	AUC = 0.894, SE = 0.050, p < 0.001, 95% CI [0.797, 0.991]	
Variables Removed From Analysis		
CSF DA	Loading < 0.3	
CSF HIAA	Loading < 0.3	
CSF HVA	Loading < 0.3	
Post DEX serum cortisol	Loading < 0.3	

Figure 6.1. Mean ± SEM rates of agonistic behavior for animals categorized as dominant (ranked 1 and 2) and subordinate (ranks 3 – 5). Dominant females received less aggressive behavior (closed circle) than those categorized as subordinate while subordinate animals emitted more submissive behaviors (open square) than dominant animals. [*From (Michopoulos et al., 2012a)*]

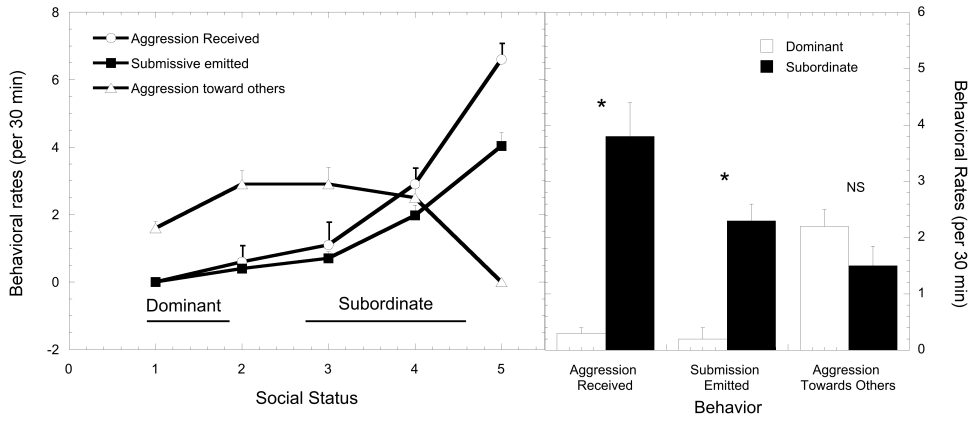


Figure 6.2. Behavioral Domain – variables retained in final DA model sorted from highest to lowest discriminant function loading (absolute values). Significance $p < 0.05$ for all variables. [*From (Michopoulos et al., 2012a)*]

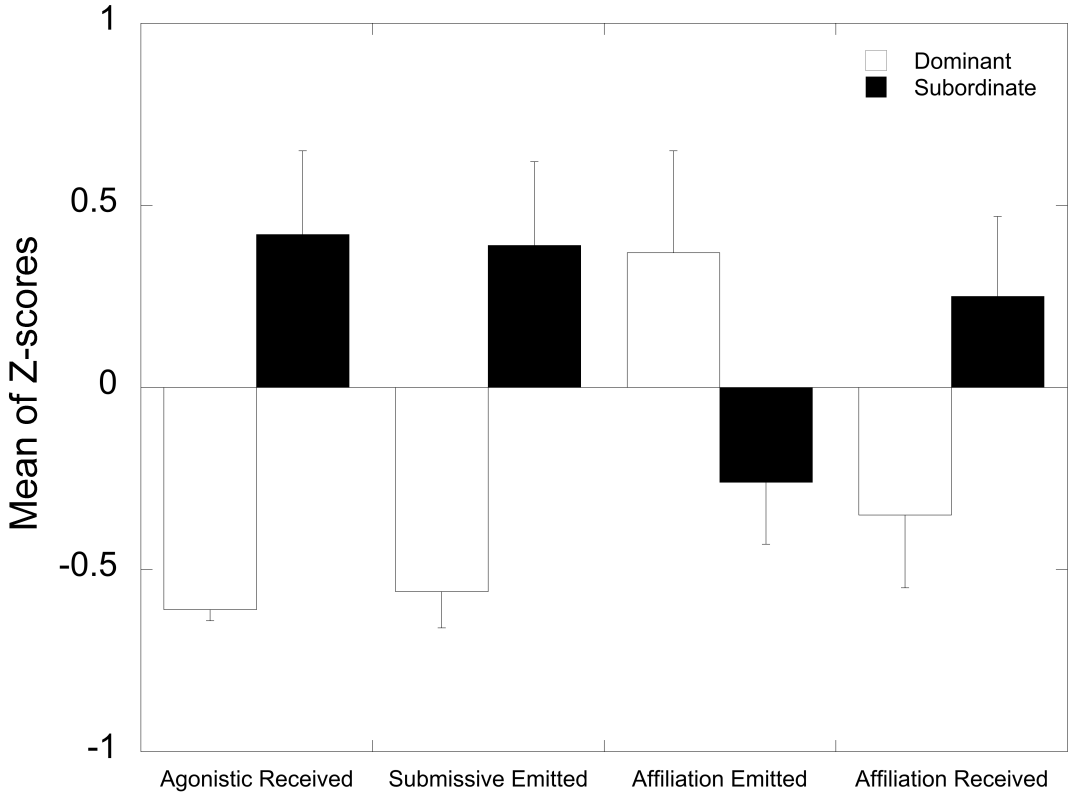


Figure 6.3. Metabolic domain – variables retained in final DA model sorted from highest to lowest discriminant function loading (absolute values). Significance $p < 0.05$ for all variables. [*From (Michopoulos et al., 2012a)*]

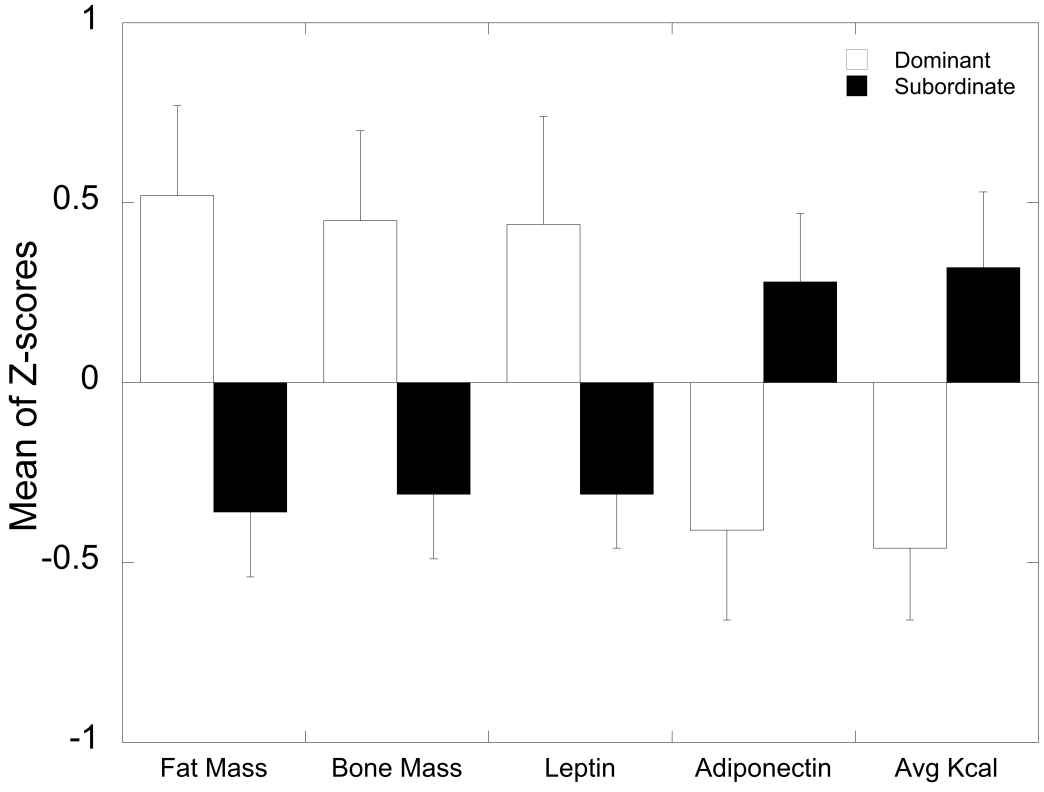


Figure 6.4. Neuroendocrine Domain – variables retained in final DA model sorted from highest to lowest discriminant function loading (absolute values). Significance $p < 0.05$ for all variables. [*From (Michopoulos et al., 2012a)*]

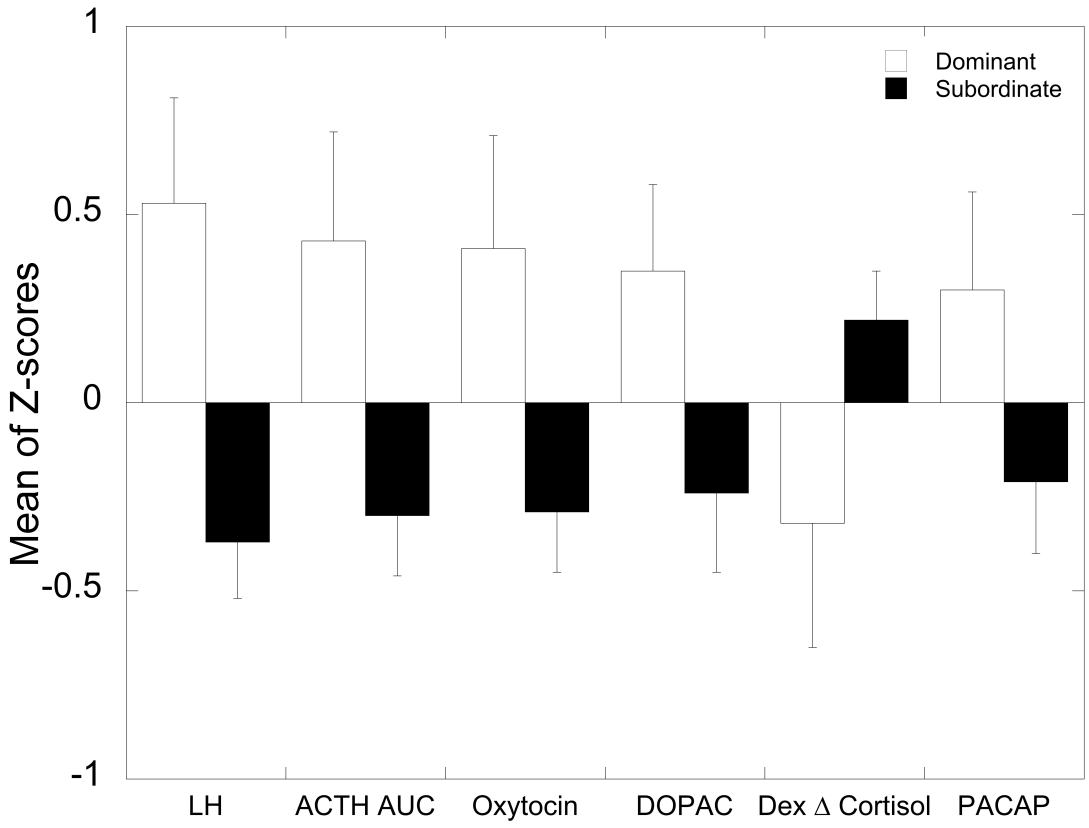


Figure 6.5. Box plots of the discriminant scores for dominant and subordinate groups in each domain (Behavioral, Metabolic, Neuroendocrine). Also listed are the effect sizes (d) comparing the scores within each domain between dominant and subordinate females. [*From (Michopoulos et al., 2012a)*]

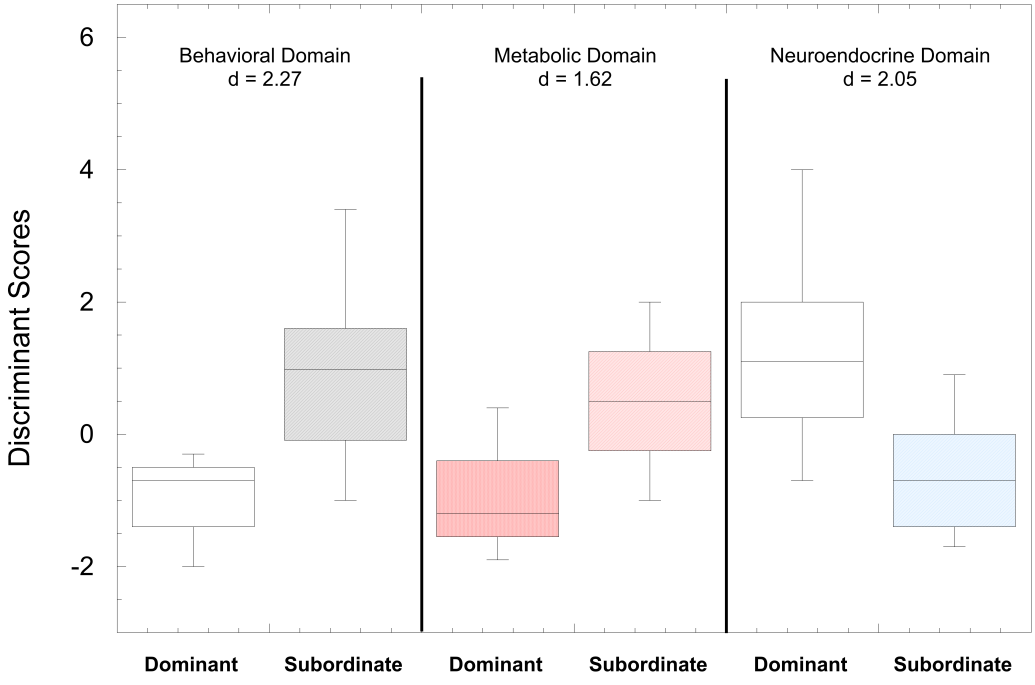
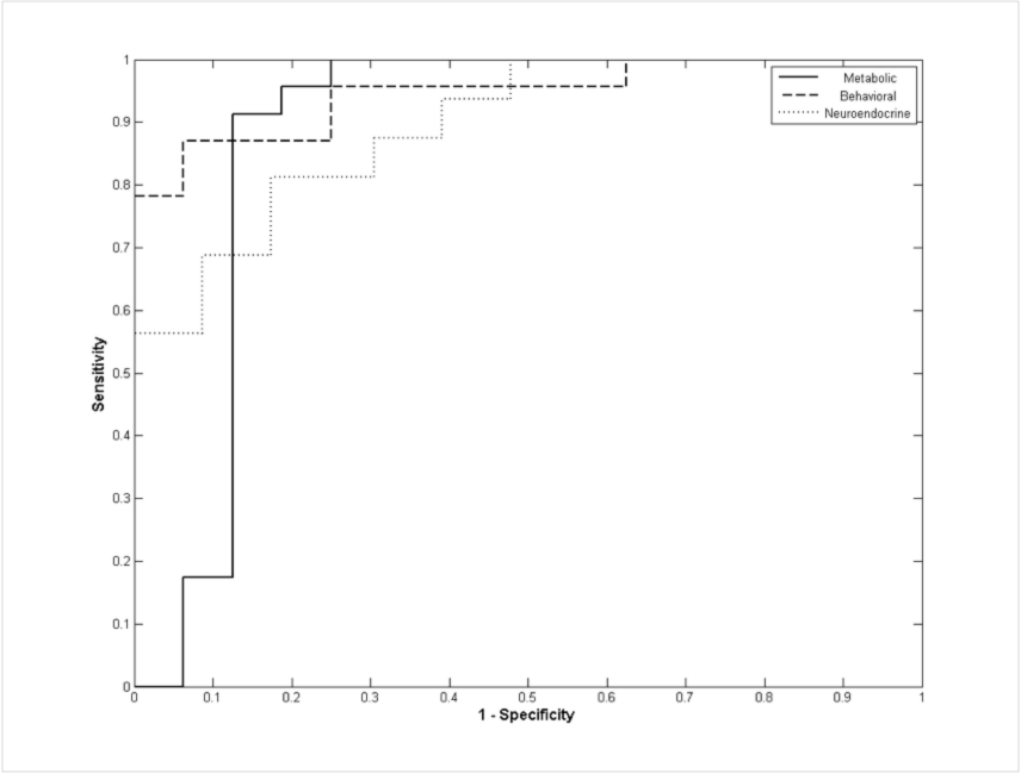


Figure 6.6. Receiver Operating Characteristic (ROC) Curves of Discriminant Analysis Scores for all 3 Domains (Positive State = Subordinate Rank) (Metabolic Solid Line, Behavioral Dashed Line, Neuroendocrine Dotted Line). [*From (Michopoulos et al., 2012a)*]



CHAPTER SEVEN:

PHYSIOLOGICAL EFFECTS OF RESTORATION OF OVARIAN CYCLICITY DUE TO COGNITIVE BEHAVIORAL THERAPY IN WOMEN WITH FUNCTIONAL HYPOTHALAMIC AMMENORHEA

7.1 Abstract

Stress-induced anovulation (SIA), often termed functional hypothalamic amenorrhea (FHA), causes reproductive compromise and infertility, and increases acute and chronic health burden in women. FHA in women most often results from psychogenic stress coupled with mild energy imbalance; as such, it represents an allostatic adaptation to circumstances unfavorable to reproduction. Similar disruptions in reproductive function are observed in macaque females subjected to the psychosocial stress of social subordination, making this an important animal model with which to study the etiology of SIA. The proximate cause of FHA in women is reduced GnRH drive that is invariably accompanied by activation of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis as evidenced by elevated cortisol levels. However, the influence of stress exposure and LHPA activity on reproductive dysfunction remains unrecognized in clinical practice as the underlying cause of reproductive compromise in women with FHA, and thus the condition it not treated appropriately. We conducted a pilot randomized clinical trial using cognitive behavioral therapy (CBT) as an intervention to alleviate problematic attitudes and reduce stressor exposure in women with FHA. We hypothesized that undergoing CBT would reduce cortisol levels and restore ovarian cyclicity in women with FHA. A 16-visit intervention over 20 weeks of CBT aimed at alleviating problematic attitudes reduced cortisol levels and restored ovarian activity. Additionally, while leptin and TSH levels were increased following CBT, levels of T3 and T4 had not recovered by the time of follow-up assessment, indicating that the thyroidal axis recovers more slowly than the LHPA and reproductive axes following CBT. Overall, our data suggest that CBT ameliorates the neuroendocrine concomitants

of FHA and highlight the notion that a cognitive nonpharmacological approach aimed at alleviating psychological attitudes impacts neuroendocrine function in women.

7.2 Stress-induced reproductive compromise

Chronic exposure to combinations of metabolic and psychosocial stressors leads to an array of adverse health consequences, including affective disorders, cardiovascular disease, osteoporosis, diabetes, infection, and reproductive compromise (Hoffman et al., 2007; Jarrell et al., 2008; Kaplan et al., 1996; Kaplan and Manuck, 2004; Marmot, 2005; Morgan et al., 2002). Whereas neuroendocrine, metabolic, and behavioral responses to acute stressors represent transient homeostatic adaptations that promote immediate survival, chronic activation of these same systems results in sustained allostatic responses that promote survival while compromising long-term health of individuals, offspring, and communities (Marmot, 2005).

Stress induces a spectrum of reproductive compromise. Both men and women can develop variable degrees of hypothalamic hypogonadism. Stress-induced anovulation (SIA) is often referred to as functional hypothalamic amenorrhea (FHA) and presents as amenorrhea in women. FHA not only causes infertility, but also increases vulnerability to other diseases and accelerates aging (Kaplan and Manuck, 1997; Kaplan and Manuck, 2004). Mechanistically, FHA can be described as a psychoneuroendocrinologic condition characterized by hypogonadism resulting from decreased drive of the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator.

Stress-induced reduction in GnRH drive occurs on a continuum and, in turn, manifests as a spectrum of reproductive compromise (Berga and Loucks, 2006). Menstrual cycle interval may be intact, short, variable, long, or absent. In women, menstrual cycle intervals may be preserved but accompanied by reduced secretion of

estradiol and progesterone from the ovary (De Souza et al., 1998; Kaplan et al., 2010). This state is often termed luteal insufficiency or hypothalamic hypogonadism. Reduced GnRH drive also may manifest clinically as polymenorrhea, oligomenorrhea, or amenorrhea. While hypothalamic hypogonadism may be clinically occult and difficult to recognize in women, it is more difficult to recognize in men unless the hypogonadism is severe enough to cause phenotypic changes such as muscle wasting, loss of libido, or decreased beard growth or infertility due to oligoasthenospermia.

The strength of GnRH drive is dependent upon the activity of the GnRH pulse generator, a collection pulsatile GnRH secreting neurons localized in the mediobasal hypothalamus that releases GnRH into the median eminence (Yen and Jaffe, 1991). GnRH induces the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland into circulation (Yen and Jaffe, 1991). LH and FSH induce steroidogenesis and gametogenesis in the ovaries (Yen and Jaffe, 1991). Synchronization of GnRH firing is critical for producing LH pulses of sufficient magnitude and for creating the GnRH surge necessary to induce the LH surge that triggers ovulation (Yen and Jaffe, 1991). Synchronization is modulated by glia cells (Witkin et al., 1997) as well as by the actions of opioids (Sapolsky and Krey, 1988) and neurosteroids (Meczekalski et al., 2000; Rasmussen, 1986; Terasawa, 1995; Thind and Goldsmith, 1989). Additionally, neuropeptide systems influence the activity of GnRH neurons in rodents and non-human primates. Kisspeptin action via the GPR54 receptor is critical for estradiol's ability to induced negative and positive feedback of LH (Hameed et al., 2011). The stress axis via corticotropin-releasing hormone (Williams et al., 1990), the inhibitory system via γ-aminobutyric acid (GABA; (Terasawa, 1994, 1995)), and the metabolic system via leptin

(Judd, 1998), neuropeptide Y (Terasawa, 1995), kisspeptin (Adachi et al., 2007; Wiegand and Terasawa, 1982), and ghrelin (Vulliemoz et al., 2008) all modulate activity of the reproductive axis.

The GnRH neurons are localized in the hypothalamus where they receive input and integrate signals from higher- and lower-order brain areas, as well as from the periphery. The hypothalamus receives input from limbic regions, including the cortex, hippocampus, and amygdala, that all play critical roles in the regulation of the LHPA axis (Herman et al., 2003). Brainstem regions, including the locus coeruleus, also connect to the hypothalamus (Swanson and Hartman, 1975). Furthermore, the blood brain barrier is fenestrated at the hypothalamus allowing signals from the periphery to communicate with hypothalamic neurons (Berglund and Sisk, 1992; Mullier et al., 2010).

Epidemiologically, the most common and important disruptions in reproduction associated with stress-induced infertility are due to a combination of psychogenic and metabolic challenges, usually in the form of psychosocial stress and negative energy balance due to over expenditure of energy and reduced caloric intake. It is critical to note that the stress-induced aberrations in fertility seen in FHA occur in women that are devoid of organic causes (Bhagavath and Layman, 2007; Kim et al., 2008) and who otherwise should be capable of producing a normal ovulatory cycle. For these reasons, the term "functional" is applied to indicate that a combination of psychogenic and metabolic challenges is responsible for anovulation and infertility, and that amelioration of these challenges results in the return of normal reproductive function (Berga, 1996).

The diagnosis of stress-induced infertility is based on the principle of exclusion, where all competing possibilities are sequentially ruled out (Warren and Fried, 2001).

Organic causes such as pituitary adenomas and hypothalamic tumors can lead to the disruption of central GnRH drive. Genetic mutations are linked to the improper migration of GnRH neurons associated with idiopathic hypogonadotropic hypogonadism and Kallmann syndrome (Caronia et al., 2011). These conditions result in the absence of spontaneous puberty and low levels of sex steroids and gonadotropins (Chan et al., 2009; Kim et al., 2008), resulting in infertility. Differential diagnosis must be made to exclude possible anovulation due to polycystic ovarian syndrome (PCOS). Women with PCOS have elevated androgens and LH levels typically comorbid with an increased incidence of obesity and hirsutism (Loucks et al., 2000), whereas women with FHA have a distinct hormone profile, including low levels of estradiol, LH, and FSH, and normal androgen levels (Berga et al., 1989).

7.2.1 Pathogenesis of stress-induced anovulation/functional hypothalamic amenorrhea (FHA)

The heightened activity of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis in women suffering from FHA not due to organic causes is the most potent confirmation that exposure to psychogenic and/or metabolic stressors impairs reproduction. Indeed, women with FHA are hypercortisolemic. Not only are levels of circulating cortisol throughout the day higher in women with FHA in comparison with eumenorrheic women (Berga et al., 1989; Suh et al., 1988) and women with other causes of amenorrhea (Berga et al., 1997), but central levels of cortisol in CSF are also elevated in women with FHA when compared to eumenorrheic women (Berga et al., 2000; Brundu et al., 2006). Furthermore, studies performing pharmacological challenges upon the LHPA axis,

including an ACTH (adrenocorticotropin) stimulation test, reveal that the adrenals of FHA women respond with higher cortisol secretion to ACTH administration than control women (Genazzani et al., 2001; Lindahl et al., 2007). In response to a corticotropin-release hormone (CRH) challenge, plasma ACTH and cortisol levels are lower in FHA women than controls (Genazzani et al., 2001; Meczekalski et al., 2000) suggesting that there is a compensatory blunting of pituitary response to CRH similar to what is seen in chronic depression (Gold et al., 1986). Even though this dysregulation of the LHPA axis is consistently described as the primary cause of FHA in women, the specific mechanisms by which this disruption in LHPA activity negatively affects reproduction remain unclear and complicated, as multiple systems are involved.

Activity of the LHPA axis directly modulates GnRH pulsatility via both cortisol and CRH. Studies in ewes show that cortisol reduces GnRH and LH pulse frequency (Oakley et al., 2008), effects that are dependent upon the background hormonal milieu as it is only seen when follicular levels of estradiol and progesterone are present (Breen et al., 2005; Oakley et al., 2009). These effects of cortisol are blocked when type II glucocorticoid receptors are antagonized (Breen et al., 2007). Substantially more work has been done looking at the myriad of ways central effects of CRH can influence the GnRH pulse generator. CRH induces release of β-endorphin from hypothalamic cells via its conversion from proopiomelanocortin (POMC), which inhibits GnRH pulsatility (Gindoff and Ferin, 1987; Sapolsky and Krey, 1988). Administration of opioid antagonists, such as naloxone, is capable of increasing LH levels in some women with FHA (Khoury et al., 1987; Quigley and Yen, 1980).

Contrary to the notion that increased CRH activity directly facilitates these changes in cortisol and effects on β-endorphin, women with FHA do not have increased levels of central CRH and have lower levels of β-endorphin than eumenorrheic women (Berga et al., 2000). Other disorders characterized by altered LHPA activity, such as depression and anorexia nervosa, show increased levels of CSF CRH (Kaye et al., 1987; Wong et al., 2000). The finding that central CRH levels are not elevated but cortisol levels are in FHA women suggests that these individuals have reduced sensitivity to glucocorticoid negative feedback because increased cortisol levels do not achieve the same degree of inhibition of CRH at the level of the hypothalamus. Taken together, the data suggest that a new allostatic set point for LHPA activity is reached in women with FHA. While CRH remains the primary central mediator of the stress axis, other neurotransmitter and neuropeptide systems play important roles in modulating CRH activity, thus complicating the view of FHA etiology. The use of selective serotonin reuptake inhibitors in the treatment of depression attenuates CRH levels (Nemeroff and Owens, 2004), suggesting serotonergic alterations in the etiology of increased LHPA drive associated with the disorder. Additionally, the inhibitory neurotransmitter GABA decreases expression of GnRH (Li and Pelletier, 1996) and decreases CRH levels (Torpy et al., 1994).

Data indicate that energy deficits also activate the LHPA axis and vice versa. Levels of metabolic signals from the periphery associated with feeding behavior are altered in FHA women, who are often characterized as having a relative energy deficit due to excessive exercise and reduced food intake (Marcus et al., 2001). Low leptin levels are a physiological marker for low fat mass and women with FHA have decreased

levels of leptin (Laughlin et al., 1998; Welt et al., 2004). This hypoleptimia does not seem to represent all females suffering from FHA, a discrepancy possibly founded in differences in how clinical subjects and controls are selected (Falsetti et al., 2002; Laughlin and Yen, 1997; Miller et al., 1998; Welt et al., 2004). Though leptin more accurately signals energy stores to the hypothalamus, ghrelin acts as a meal-initiating signal upon these same hypothalamic regions (Wynne et al., 2004) and thus more closely follows feeding behavior than leptin. Women with FHA have increased levels of ghrelin (Schneider and Warren, 2006), results consistent with other findings in individuals with eating disorders and/or negative energy deficits (Leidy et al., 2004; Tanaka et al., 2002; Tolle et al., 2003). While these results suggest that either of these metabolic signals could trigger downstream changes in the HPG axis associated with FHA, it remains uncertain as to whether this is the case, as the altered values of leptin and ghrelin still fall within the normal range (Genazzani et al., 2001; Lindahl et al., 2007; Meczekalski et al., 2000).

Activation of the LHPA axis and the resulting release of cortisol, a glucocorticoid, mobilize fat stores and gluconeogenesis, providing sufficient energy for an organism to flee from harm. Cortisol also acts to induce a hypothyroid state by decreasing the activity of the hypothalamic-pituitary-thyroidal (HPT) axis and decreasing levels of thyroid stimulating-hormone (TSH) (Samuels et al., 1994; Samuels and McDaniel, 1997). Interestingly, women with FHA have reduced levels of thyronine (T3) and thyroxine (T4) even though levels of TSH remain within the normal range (Berga et al., 1989). Thus the hypothalamic set point for TSH is altered in FHA, as low levels of T3 and T4 would normally induce an increase of TSH. This hypothalamic hypothyroidism concurrent with

the hypothalamic hypogonadism in FHA women suggests that the hypothalamus is the critical final common pathway, as it communicates with higher order areas of the brain and the external environment to maintain homeostasis during threatening situations (Berga, 2008). Similar allostatic alterations to hypothalamic set points critical for reproductive and metabolic functioning are associated with FHA, but what remains to be known is whether it is the primary disturbance in the activity of the LHPA axis or that of the HPT axis that triggers changes in hypothalamic drive. However, exposure to psychogenic stress in the presence of energy deficiency elicits adverse effects on the function of the reproductive axis (Petrides et al., 1994; Williams et al., 2007).

7.2.2 Treatment of FHA with cognitive behavioral therapy

Importantly, FHA is a reversible form of amenorrhea whose proximate cause is reduced GnRH drive that is invariably accompanied by increased LHPA activation as evidenced by elevated circulating and cerebrospinal fluid levels of cortisol (Berga et al., 1989; Suh et al., 1988). Furthermore, previous studies that exclude women who meet criteria for depression, eating disorders, or other psychiatric disorders show that women with FHA report greater levels of perfectionism, a higher need for social approval and altered eating attitudes than eumenorrheic women (Giles and Berga, 1993; Marcus et al., 2001). These two factors indicating that women with maladaptive attitudes and subtle metabolic disturbances suggested to Berga et. al. that a behavioral intervention targeted at these problematic attitudes might restore hypothalamic function and thus ovarian cyclicity in women with FHA (Berga et al., 2003). To test this hypothesis, a 16-visit cognitive behavioral therapy (CBT) regimen was implemented and administered to newly

diagnosed women with FHA in a randomized fashion to assess if ovarian function would return following CBT intervention (Berga et al., 2003).

An interim analysis showed that CBT intervention in women with FHA restored ovarian cyclicity compared to the control observation-only arm of the study (Berga et al., 2003). Women randomized to observation-only or to CBT had similar ages and body mass indices at enrollment as well as at the end of follow up after the interventions had occurred. CBT restored estradiol (E2) and progesterone (P4) levels in women with FHA at a higher rate than observation-only (87.5% vs. 25% respectively).

7.2.3 Overall rationale.

The neuroendocrine concomitants of FHA in women may provide a clue as to the pathogenesis of the stress-induced disorder. It remains uncertain whether CBT in women with FHA was capable of affecting the neuroendocrine aberrations characteristic of FHA in women, specifically whether CBT resulted in the recovery of the LHPA and HPT axes similarly. Using samples from the original CBT study, we assessed the extent to which neuroendocrine aberrations seen in FHA women are reversed by CBT. We hypothesized that cortisol in women with FHA who received CBT would decrease following treatment compared to those women who were observed only. Furthermore, we hypothesized that the HPT axis would show partial recovery in response to CBT as it is the last axis to recovery following operative and injury stressor exposure (Barton, 1987; Spratt et al., 1993; Spratt et al., 2008).

7.3 Materials and Methods

As previously reported, the diagnosis of FHA in women was established by carefully screening women with amenorrhea to exclude organic and functional causes of anovulation as well psychiatric and eating disorders (Berga et al., 2003). Inclusion criteria for the study included an ideal body weight greater than 90%, weight loss < 10 lbs, and a day-awake, night-rest schedule (Berga et al., 2003). Seventeen women met all inclusion criteria and exhibited none of the exclusion criteria, such as organic forms of amenorrhea, premature menopause, drug use and thyroid disorders. After enrollment, all subjects were followed for four weeks to collect baseline hormone profiles. Subjects were then admitted to a General Clinical Research Center (GCRC) and blood samples were collected at 15-minute intervals for 24 hours via an indwelling intravenous catheter to assess levels of cortisol, TSH, free T3, free T4, total T3, total T4 and leptin.

A diagnosis of FHA was confirmed if women exhibited persistent amenorrhea during the baseline four-week screening period, progesterone levels lower than 5 ng/mL, and LH pulse frequency was less than 10 pulses over the course of 24 hours (Berga et al., 1997; Berga et al., 2003). A trained psychologist conducted a formal structured interview to exclude mental disorders and then subjects were randomized to either CBT or observation-only (Berga et al., 2003). Women randomized to observation were offered CBT after their observation period. Nine women were randomized to the observation arm of the study and eight women to a 16-session CBT over a 20-week period. Sessions 1-6 of CBT focused on establishing healthy eating patterns, sessions 7-12 identified maladaptive attitudes about eating and weight and focused on adopting a healthy lifestyle, and sessions 13-16 prepared subjects for the termination of CBT (Berga et al.,

2003). The Institutional Review Board of Magee-Women's Hospital and the University of Pittsburgh approved all procedures and the study protocol.

Blood samples were collected every week for E2 and P4 analysis before and during the final 6 weeks of CBT or observation to assess ovarian activity (Berga et al., 2003). Estradiol (E2) and progesterone (P4) were measured using a RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). Subjects were asked to report episodes of vaginal bleeding. Any reports of bleeding during CBT or observation were followed up with a blood sample 21 days later to assess whether ovulation had occurred. Full recovery occurred when definite evidence of ovulation by E2 levels >100 pg/mL and P4 levels >5 ng/mL was observed. Partial recovery was defined as E2 levels >60 pg/mL and P4 levels <5 ng/mL and unrecovered as E2 <60 pg/mL and P4 < 5ng/mL (persistent anovulation) (Berga et al., 1997; Berga et al., 2003).

At the cessation of either CBT or observation, subjects were again admitted into the GCRC where samples were drawn at 15-minute intervals for 24 hours to assess daily fluctuations and levels of cortisol. Leptin and TSH was measured hourly over the course of those 24 hours, while a single measure of free T3, free T4, total T3, total T4 was assessed from the initial sample following participation in the study. Cortisol and free and total T3 and T4 were analyzed by radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) (Berga et al., 1997). Leptin (Linco) (Mancini et al., 2005) and TSH (Nichols Institute) (Berga et al., 1997) were measured by immunoradiometric assay.

Statistical Analyses. As described in the initial report (Berga et al., 2003), randomization was performed via a permuted block design, with blocks of varying sizes.

Data were summarized as mean ± standard error of the mean (SEM). An intention to treat (ITT) analysis was performed; the main and interaction effects of treatment (CBT vs. observation) and time (pre-treatment vs. post-treatment) on cortisol, TSH, T4, T3, free T4, free T3, leptin, and body weights were evaluated with analysis of variance for repeated measures (RM-ANOVA). All analyses were done using SPSS version 19. Test results with a $p \leq 0.05$ were considered significant and post-hoc analysis conducted when necessary by paired t-tests.

7.4 Results

Women with FHA who were randomized to the CBT arm exhibited a higher rate of ovarian recovery (87.5%) than women with FHA who were randomized to the observation arm (33.3%) ($\chi^2 = 5.13$, $p = 0.02$). The odds ratio for ovarian recovery on CBT as opposed to observation was 14, with a 95% confidence interval of 1.14 to 172.6 ($p = 0.01$). Of the women who underwent CBT, six resumed ovulating, one exhibited partial recovery, and one showed no recovery. Of the women who underwent observation, three exhibited partial recovery and six remained anovulatory.

A RM-ANOVA detected a significant effect of time on nocturnal cortisol levels ($F_{1, 15} = 9.45$, $p=0.008$), as cortisol levels following participation in the study were decreased compared to baseline (95.9 ± 7.6 vs. 85.9 ± 6.4 respectively). However, this main effect of time interacted with treatment ($F_{1, 15} = 3.32$, $p=0.088$), as cortisol levels were reduced after CBT but not following observation ($p = 0.006$; Figure 7.1).

Body weights were not affected by treatment or observation over the course of the study ($F_{1, 15} = 0.53$, $p=0.48$; Figure 7.2A). However, overall leptin levels were

significantly affected by a time by treatment interaction ($F_{1, 15} = 6.73$, $p=0.02$). Leptin levels in women who underwent CBT were increased ($p = 0.09$), whereas leptin levels stayed the same in women who underwent observation (Figure 7.2B).

Additionally, TSH levels showed a significant treatment by time interaction ($F_{1, 15} = 5.41$, $p=0.034$). TSH levels in women who underwent CBT increased following therapy ($p = 0.009$) whereas TSH levels did not change in women who underwent observation (Figure 7.2C). Levels of free T3 (Figure 7.2D), total T3, free T4, and total T4 did not change by time, treatment or interaction ($p > 0.05$; Table 7.1).

7.5 Discussion

We previously reported that women with FHA who underwent CBT were more likely to recover ovarian activity than women with FHA who were observed (Berga et al., 2003). We now report that cortisol levels in women randomized to CBT were reduced whereas cortisol levels in women randomized to observation remained unchanged. The CBT–treated group displayed concomitant increased leptin and TSH levels while weight, T3, free T3, T4, and free T4 remained unchanged in either group. The lack of T3 and T4 increases in the presence of increased TSH in the CBT-treated group indicates partial recovery of the hypothalamic-pituitary-thyroidal axis. Together, our data suggest that CBT ameliorates the neuroendocrine concomitants of FHA and results in ovarian recovery. Our findings highlight the role of nonpharmacological, psychological approaches in ameliorating neuroendocrine aberrations in women with FHA and potentially other manifestations of stress related reproductive compromise associated with heightened activity of the LHPA axis.

The reduction in cortisol levels following CBT coincident with resumption of ovarian activity supports the notion that hypercortisolism (Berga et al., 1989; Brundu et al., 2006; Suh et al., 1988) and dysregulation of the LHPA axis (Genazzani et al., 2001; Lindahl et al., 2007; Meczekalski et al., 2000) characteristic of women with FHA are causally related to the reduction in GnRH drive and other neuroendocrine concomitants as well as the reproductive dysfunction and resulting infertility. A direct role of stress hormones in the etiology of an FHA phenotype is suggested by pharmacological studies, as exogenous administration of cortisol reduced LH pulse frequency during the follicular phase in normal women (Saketos et al., 1993). Similarly, a pharmacological elevation of cortisol suppressed LH levels in female rhesus monkeys (Michopoulos et al., 2009a). Cortisol also reduced GnRH and LH pulse frequency in ewes (Oakley et al., 2008) via activation of type II glucocorticoid receptors (Breen et al., 2007). Thus, the CBT-induced decrease in cortisol levels in women with FHA supports the notion that recovery of ovarian activity is linked to restoration of the LHPA axis akin to what is seen in spontaneous resumption of menses in women with previous FHA (Berga et al., 1997).

Women with FHA report concomitant psychogenic challenge combined with behaviors that induce intermittent energy deficits and represent metabolic stressors (Berga et al., 1997; Warren et al., 1999). The constellation of psychogenic and metabolic factors necessitates metabolic adaptation including alterations in the appetite signaling systems and the hypothalamic-pituitary-thyroidal (HPT) axis. While CBT did not alter body weight, it did increase leptin levels. This data suggests that CBT focused on resolving problematic attitudes was successful in reversing the relative energy deficit described in women with FHA due to exercise and altered feeding behavior (Laughlin et

al., 1998; Marcus et al., 2001; Welt et al., 2004). It must be noted that while exogenous leptin administration has been shown to restore ovarian function in some women with amenorrhea (Welt et al., 2004), our findings suggest that restoration of LHPA activation permits partial metabolic recovery as evidenced by the rise in leptin levels. Thus, the low leptin levels reflect rather than cause FHA.

Further evidence to suggest that CBT had beneficial effects on metabolism was the increase in TSH levels following CBT treatment. Whether of endogenous or exogenous origin, glucocorticoids blunt the thyroid-releasing hormone (TRH) to TSH signal and alter HPT feedback, thus decreasing the amount of TSH, T3, and T4 in circulation in an effort to conserve energy (Samuels et al., 1994; Samuels and McDaniel, 1997). Women with FHA display the same pattern of hypothalamic hypothyroidism (Berga et al., 1989). The decrease of cortisol following CBT and the concurrent rise in TSH levels in these women indicates that the hypothalamic setpoint is altered following CBT. The hypothalamus registers that levels of T3 and T4 are low following the diminution of cortisol and acts to increase levels of T3 and T4 by increasing the TSH signal, presumably by increasing TRH release from the hypothalamus or by increasing the TSH response to TRH.

The current data indicate that women who recovered ovarian activity following CBT exhibited only partial recovery of HPT function by the time of the second assessment, as levels of free T3 and free T4 remained low in women who underwent CBT. Similarly, partial recovery of HPT function occurs in women who undergo spontaneous resumption of ovarian activity in the presence of LHPA recovery (Berga et al., 1997). While this partial recovery of the HPT axis following CBT and decreased

cortisol levels could indicate that T3 and T4 levels are unaltered by CBT, a more parsimonious answer is that the time of sampling at follow-up was not sufficient to allow for full recovery from hypothalamic hypothyroidism. We expected a delay in the full recovery of the thyroidal axis following CBT because prior studies suggested that hypothalamic hypothyroidism associated with exposure to stressors is the last axis to recover following cessation of the stressor. Indeed, men who undergo surgery or physical stress become hypothyroidal and levels of T3 and T4 are the last to recover following stressor alleviation and decreased cortisol levels (Barton, 1987; Spratt et al., 2008).

Together, our data suggest that a CBT intervention restores ovarian activity in women suffering from FHA (Berga et al., 2003) and decreased cortisol levels and increased TSH accompany that ovarian recovery. The ability of CBT to alter neuroendocrine setpoints indicates that nonpharmacologic psychological approaches designed to address problematic attitudes that activate the LHPA axis are potent tools for treating FHA in women. The effect size of cognitive interventions accrues with time because they induce learning that allows individuals to manage maladaptive attitudes and thereby decrease stress levels. Mitigating the effects of the LHPA axis and resolving the proximate cause of reproductive dysfunction in women with FHA also reverses hypoestrogenism (Berga et al., 2003) that can lead to adverse cardiovascular, skeletal, and cognitive consequences (Berga and Loucks, 2005, 2006; Marcus et al., 2001).

Currently, the most popular approach for treating women with FHA is to offer hormone replacement for those not seeking pregnancy or ovulation induction for those who do (Hurley et al., 1984; Miller et al., 1983) (Berga and Loucks, 2006). While

pharmacologic approaches make up for anovulation, they do not address the underlying etiology of the disorder, namely the psychoneuroendocrinologic alterations that are triggered by stressful environments. Without addressing the attitudes and behaviors that sustain LHPA activation, the neuroendocrine concomitants persist and likely exact adverse acute and chronic health consequences. Ovulation induction in the presence of neuroendocrine allostasis is predicted to adversely affect the development of offspring. The hormonal induction of ovulation in amenorrheic women is linked to a higher risk of premature labor as well as decreased body mass in offspring (van der Spuy et al., 1988), suggesting that maternal gestational psychosocial or metabolic distress that is not resolved triggers adverse events on fetal development. Indeed, hypothyroidism in pregnant rats results in altered neuronal migration and fetal development of the hippocampus and cortex (Auso et al., 2004; Lavado-Autric et al., 2003). Furthermore, background stress levels in women with FHA may impair their parenting skills and adversely affect their child's psychosocial, physiological and intellectual development (Haddow et al., 1999; Pike and Rodin, 1991).

The potential for epigenetic effects due to maternal stress exposure during gestation on offspring wellbeing make it critical for clinicians to address the etiological basis for FHA as opposed to simply treating the primary outcome directly, as neglecting to do this adversely effects future generations. The best way to effectively treat FHA in women is to manage the psychogenic challenges that activate the stress axis. Stress reduction allows for reversal of hypothalamic allostasis and restores ovarian function. Behavioral therapies for women with FHA revolve around determining and alleviating the sources of psychogenic and metabolic stressors in their lives. Concurrent with

providing appropriate emotional support, these interventions teach women self-care via stress management, relaxation and psychoeducation (Berga and Loucks, 2006). Our data indicate that CBT in women with FHA is a potent way with which to rescue ovarian function by treating the cause of the condition (Berga et al., 2003).

The success of behavioral therapy in ameliorating the behavioral responses to environmental stressors underscores how individual affect, behavior, and personality can influence neuroendocrine function. Importantly, genetics can influence all these factors and interact with the environment to exacerbate the adverse behavioral and physiological effects of stressful situations. Variation at genetic loci important for the functioning of the serotonergic system (Albert and Lemonde, 2004; Caspi et al., 2003; Cowen et al., 1994; Hansenne et al., 2002; Lesch and Gutknecht, 2004; Savitz et al., 2009), stress axis (Claes, 2009; van Rossum et al., 2002) and the actions of estrogen influence individual responses to environmental stressors (Michopoulos et al., 2009a; Michopoulos et al., 2011b) and thus can exacerbate the incidence of stress-induced disorders. While FHA is a stress-induced disorder, at the current time, there is a lack of association studies linking genetic polymorphisms with individual vulnerability to stress-induced deficits in reproduction in women. Understanding the role of genetics in meditating individual vulnerability to stress-induced disruptions in fertility would allow for a more personalized and effective method for treating women with FHA, allowing clinicians to determine which women would optimally benefit from CBT. This method could be combined with a pharmacogenetic approach to assess which women would benefit from pharmacological intervention concurrent with CBT.

Table 7.1. Mean ± SEM values for free T3, total T4, and total T3 according to treatment arm (observation vs. CBT) and before (pre) and after (post) treatment. A RM-ANOVA showed that none of these endpoints were affected by time, treatment or their interaction ($p > 0.05$).

	Observation		**CBT**	
Measure	Pre	Post	Pre	Post
Free T4 (ng/dL)	0.86 ± 0.07	0.85 ± 0.06	0.79 ± 0.07	0.78 ± 0.06
Total T3 (pg/dL)	71.6 ± 5.21	82.9 ± 4.82	61.2 ± 4.82	67.3 ± 6.63
Total T4 (ng/dL)	5.64 ± 0.35	5.41 ± 0.31	5.31 ± 0.33	5.33 ± 0.28

Figure 7.1. Mean ± SEM levels of nocturnal (2400-0800) cortisol levels by treatment arm (observation vs. CBT) and before (pre) and after (post) treatment. Asterisk denotes significant reduction in cortisol levels in women with FHA who recovered following CBT ($p < 0.05$).

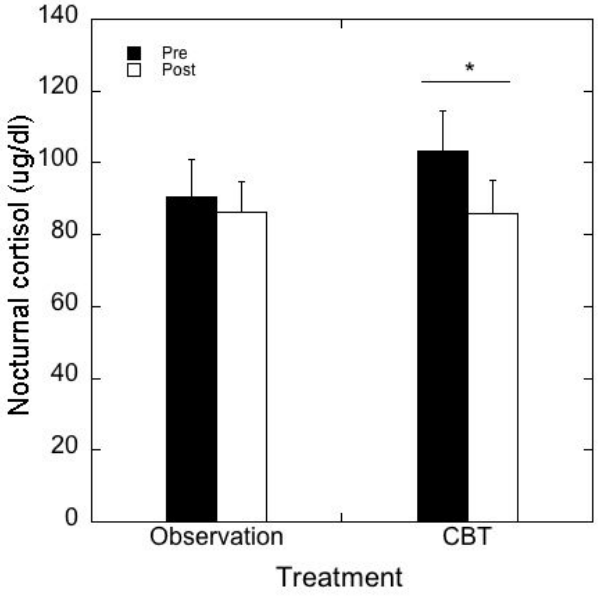
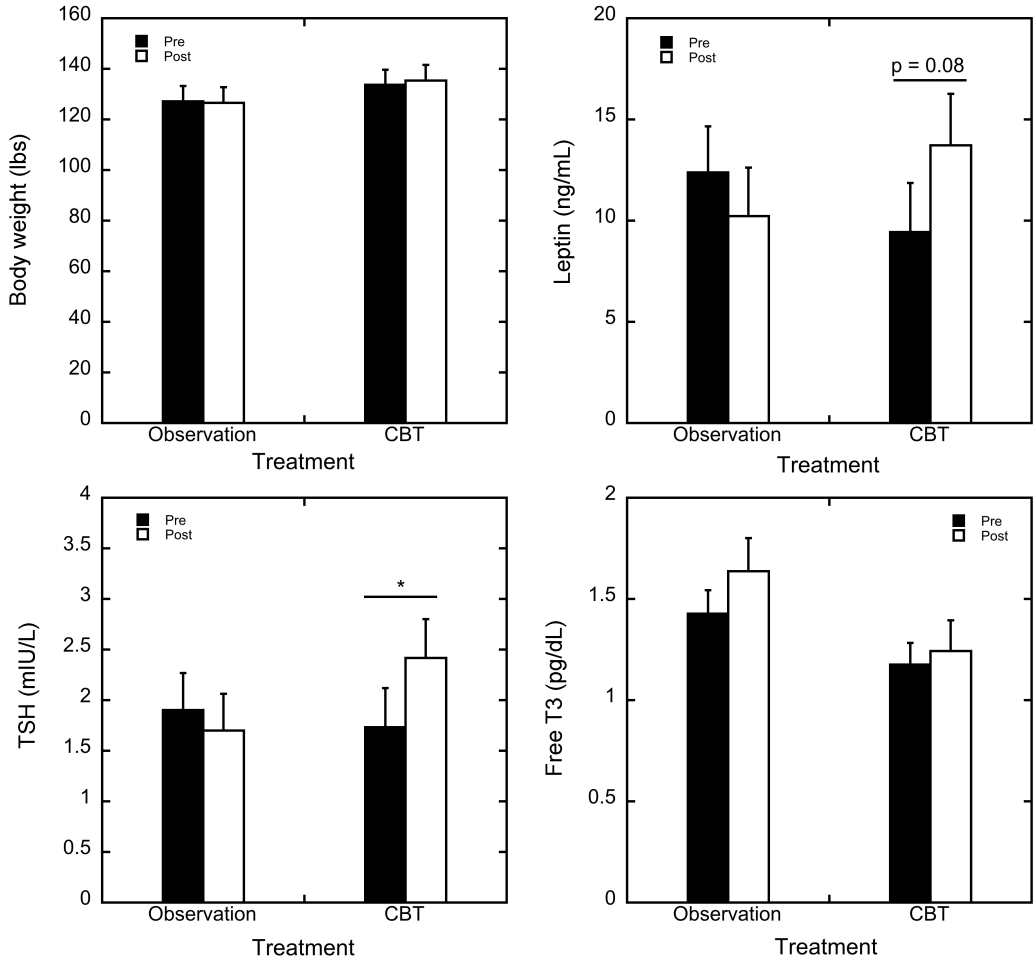


Figure 7.2. Mean ± SEM of (A) body weight, (B) leptin, (C) TSH, and (D) free T3 in women who underwent observation or CBT at the beginning (pre) and following (post) treatment. Asterisk denotes significant increase in overall TSH levels in women with FHA who recovered following CBT ($p < 0.05$).



CHAPTER EIGHT:

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