INDIVIDUAL DIFFERENCES IN ADRENAL AXIS ACTIVITY AND STRESS SENSITIVITY IN FEMALE CYNOMOLGUS MONKEYS (*MACACA FASCICULARIS*)

by
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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of
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ABSTRACT

In women, one of the most common forms of infertility is known as 'stress-induced amenorrhea', thought to be caused by a combined effect of mild psychosocial stress + metabolic stress acting to suppress gonadotropin-releasing hormone (GnRH) drive to the reproductive (HPG) axis and inhibit normal reproductive function. Women with this disorder report a greater degree of psychosocial stress in their lives as compared to healthy women, although they do not experience more stressful life events. They also tend to diet and exercise in an attempt to control stress. It thus appears that stress-induced amenorrhea occurs in women who have an increased *sensitivity* to everyday life stresses. Cortisol has been reported to be elevated in this patient population, and activation of the hypothalamic-pituitary-adrenal (HPA) axis has been suggested as a potential neural mechanism underlying the etiology of stress-induced amenorrhea. Our laboratory has developed a non-human primate model of sensitivity to stress-induced reproductive dysfunction based on clinical descriptions of the stressors experienced by this clinical population. Female monkeys, when exposed to mild combined psychosocial + metabolic stress (i.e. move to a novel room + a 20% reduction in available calories), can be categorized as highly stress-resilient ("HSR"; maintain normal menstrual cycles in response to stress), medium stress-resilient ("MSR"; slowly become anovulatory in response to stress), or stress-sensitive ("SS"; rapidly become anovulatory in response to stress). Our laboratory has shown that SS monkeys have increased expression of corticotropin-releasing hormone (CRH) in the caudal regions of the paraventricular nucleus (PVN). CRH provides the central neural drive to the HPA axis. To test the hypothesis that elevated activity of the HPA axis is a primary neural mechanism

underlying the sensitivity to stress-induced reproductive dysfunction, I conducted physiological, pharmacological and immunocytochemical studies in two different groups of female cynomolgus monkeys (*Macaca fascicularis*). For all studies in Chapters 2 and 3, monkeys were fitted with indwelling catheters that allowed undisturbed collection of blood samples and infusion of drugs for physiological characterization and pharmacological manipulation of HPA axis activity. SS monkeys did not differ from HSR or MSR monkeys in adrenocorticotropic hormone (ACTH) or cortisol secretion over a normal day, in ACTH or cortisol response to an acute psychological stressor, in cortisol response to dexamethasone negative feedback, in concentration of cortisol found in hair, or in adrenal weight. However, when exposed to the specific condition of mild psychosocial + metabolic stress, monkeys that became anovulatory in response to this mild combined stress (MSR+SS monkeys) had an increase in daytime cortisol release. These findings suggested that greater activation of the HPA axis in response to everyday life stresses may be playing a role in sensitizing animals to stress-induced reproductive dysfunction. I next performed a pharmacological study to test whether a blockade of stress-induced activation of the HPA axis could prevent stress-induced suppression of the reproductive axis. Monkeys were exposed to mild combined stress and the immediate effects of stress exposure on pulsatile LH secretion were studied. Monkeys that became anovulatory in response to stress (MSR+SS monkeys) showed a significant suppression of pulsatile LH secretion when stressed, whereas HSR monkeys did not. Treatment with the specific CRH-R1 antagonist, antalarmin, prevented the stress-induced suppression of LH pulse frequency in monkeys that became anovulatory in response to stress (MSR+SS), without blocking the stress-induced increase in cortisol. These findings

indicated that activation of the HPA axis was not causing acute suppression of reproductive function in stress-sensitive individuals, but that CRH acting through another system besides the HPA axis appears to be involved in stress-induced suppression of the reproductive axis in MSR+SS animals. In Chapter 4, immunocytochemical staining of CRH-positive neural fibers was examined in brain tissue previously collected from a second group of animals in the early follicular phase of a control, non-stressed menstrual cycle. Our laboratory had previously shown that SS monkeys have lower release of serotonin in the brain and suppressed expression of serotonin-related genes in the dorsal raphe nucleus, the central site of serotonin production in the brain. Therefore, I chose to examine CRH neuronal input to the raphe nucleus, as CRH signaling is inhibitory to the serotonin neurons in this region. The area of CRH immunopositive fiber staining was greater in both the dorsal and median raphe nucleus of SS animals compared to HSR animals, indicating that the increased CRH expression in SS monkeys may be interacting with serotonin to affect reproductive function. The results of this dissertation suggest a new direction for research directed towards improving our understanding of the etiology of stress-induced reproductive dysfunction would be to examine the possible interactions between CRH and serotonin neurotransmitter systems.

Chapter 1

INTRODUCTION

1.1 The Hypothalamic-Pituitary-Gonadal (HPG) Axis and the Female Menstrual Cycle

Reproductive function in females is an intricate physiological process, controlled by neurons generating the central drive to the reproductive organs (regulated by multiple neurotransmitter and neuropeptide systems), and hormones secreted by the anterior pituitary and the gonads [i.e., the hypothalamic-pituitary-gonadal (HPG) axis; Freeman, 1994; Hotchkiss and Knobil, 1994]. Gonadotropin releasing hormone (GnRH) neurons produce the peptide GnRH and release it into the hypophyseal portal system, which connects the hypothalamus and pituitary, in a pulsatile manner (Figure 1.1; for review see Steiner & Cameron, 1989). The mechanism leading to the coordinated release of GnRH from neuronal axon terminals into fenestrated capillaries of the median eminence to supply distinct pulses of GnRH into the portal bloodstream remains unknown at this time. However, when pulses of GnRH reach the anterior pituitary, GnRH binds to membrane receptors on the pituitary gonadotrophes and stimulates the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are also released into the peripheral circulation in a pulsatile manner. In turn, the gonadotropins stimulate the release of sex steroid hormones from the gonads. In the female, the ovaries produce and release estradiol (E_2), progesterone (P_4), and inhibin in response to LH and FSH. In males, pituitary hormone stimulation of the testes results in the secretion of testosterone (T_4) and inhibin.

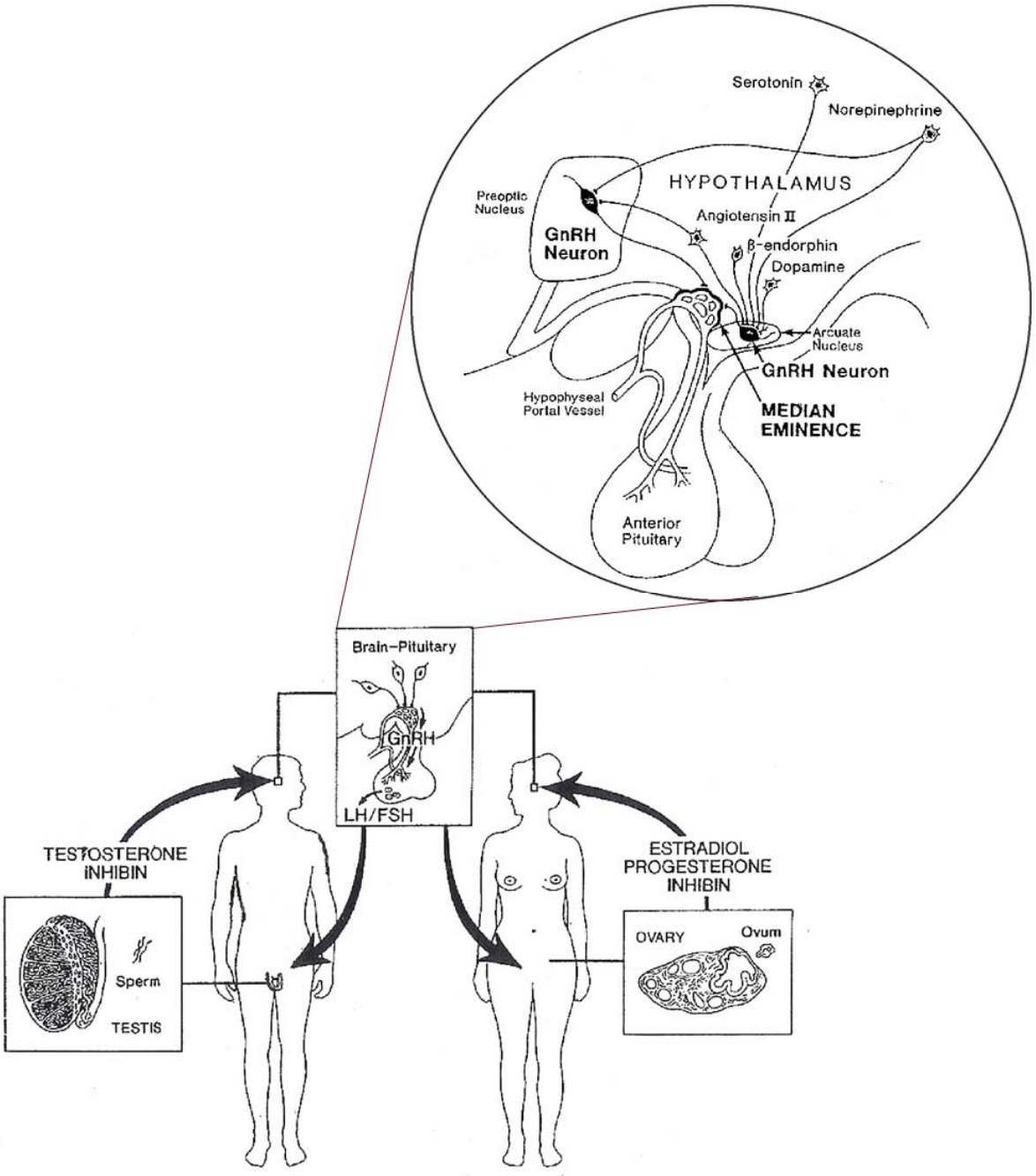


Figure 1.1 The hypothalamic-pituitary-gonadal (HPG) axis and examples of neurotransmitter interactions (inset) mediating the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (adapted from Steiner & Cameron, 1989).

Dierschke and colleagues (1970) first observed the pulsatile nature of GnRH secretion in the ovariectomized rhesus macaque (*Macaca mulatta*), and it has since been reported in many other species including the rat (Gay & Sheth, 1972), mouse (Coquelin & Bronson, 1980), guinea pig (Donovan et al., 1977), rabbit (Rowe et al., 1975), chicken (Wilson & Sharp, 1975), cat (Johnson & Gay, 1981), sheep (Butler et al., 1972; Katongole et al., 1974), pig (Foxcroft et al., 1975; Brinkley, 1981), cow (Katongole et al., 1971; Rahe et al., 1980), and human (Nankin & Troen, 1971; Yen et al., 1972). Each burst of gonadotropin from the pituitary is a direct consequence of a GnRH pulse from the hypothalamus (Carmel et al., 1976; Clarke & Cummins, 1982; Knobil, 1989). This was first demonstrated with simultaneous measurement of GnRH in blood samples collected from the portal bloodstream and LH in blood samples collected from the peripheral circulation (Dierschke et al., 1970). As each pulse of LH from the pituitary is preceded by a pulse of GnRH, the network of GnRH neurons in the hypothalamus has been referred to as the "hypothalamic pulse generator" (Goodman & Karsch, 1981; Knobil, 1981). In both the macaque and human, the pulse generator has been localized to the arcuate nucleus of the mediobasal hypothalamus (Plant et al., 1978; Ule et al., 1984). In contrast, these GnRH neurons in most non-primate species are located more rostrally in the medial pre-optic area of the hypothalamus (Dobson et al., 2003). Species differences in final location of GnRH neurons arise during embryonic development, as GnRH neurons originate outside the brain in the nasal placode and migrate to species-specific locations (Dobson et al., 2003). In addition to similarities in the physiological regulation of reproductive function in primate species, this anatomical distinction makes

the macaque a particularly useful model for studying neural mechanisms regulating GnRH secretion in women.

The *pulsatile* release of GnRH from the hypothalamus plays an extremely important physiological role. Pulsatile, but not tonic, administration of exogenous GnRH is required for the restoration of physiologic secretion of gonadotropins in the pre-pubertal macaque as well as in macaque models with an experimentally-induced GnRH deficiency (Belchetz et al., 1978; Knobil, 1980; Wildt et al., 1980). Furthermore, the frequency of pulsatile GnRH is also critical, with biological "thresholds" for menstrual cycle induction. In monkey models of complete GnRH depletion, hypothalamically lesioned rhesus monkeys have no stimulation of the reproductive axis. In order to resume ovulation, exogenously administered GnRH must be given at a frequency of no less than once every 90 minutes (Pohl et al., 1983). A similar requirement for pulsatile GnRH secretion has been shown in humans in order to induce puberty and ovulation in GnRH deficient patients (Leyendecker et al., 1980; Crowley et al., 1980; Hoffman et al., 1982).

As secretion of GnRH is necessary for activation of the HPG axis, it would be of interest to measure the frequency, amplitude, and other characteristics of GnRH secretion as a measure of central drive to the reproductive axis in individuals with reproductive dysfunction. However, there are several difficulties with the accurate and reliable collection of such data. GnRH is a 10 amino acid peptide with a rapid (i.e., 2 to 4 minute) half-life (Arimura et al., 1974; Pimstone et al., 1977; Barron et al., 1982). Moreover, cannulating and collecting portal blood is technically very difficult (Dierschke et al., 1970). These difficulties lead most investigators to use detection of LH pulses as inferential measurements of GnRH secretion. As GnRH stimulation of the anterior

pituitary results in pulsatile gonadotropin (LH and FSH) secretion into the peripheral circulation (Carmel et al., 1976; Clarke & Cummins, 1982; Knobil, 1989), the frequency and amplitude of pulsatile LH in peripheral blood are the most widely used measures of central drive to the HPG axis (Crowley et al., 1985).

In contrast to GnRH, LH and FSH are relatively large protein hormones. The structure of LH and FSH are quite similar, sharing an identical alpha polypeptide chain but differing with respect to their unique beta chains (Steiner & Cameron, 1989). FSH has a significantly greater number of carbohydrates associated with its molecular structure, resulting in a molecular weight that is heavier than that of the LH molecule. As such, FSH has a much longer biologic half-life (3 to 4 hours) compared to LH, which has a half-life of only 20 minutes (Amin & Hunter, 1970; Pepperell et al., 1975). Although both hormones are released in a pulsatile manner, blood concentrations of FSH are less variable and more stable than those of LH. Conversely, the fast clearance rate of LH provides a much clearer and more reliable assessment of pulsatile GnRH activity than does FSH (Crowley et al., 1985).

Frequency and amplitude of LH secretion from the anterior pituitary vary widely between species, over time, and within a given patient population (Crowley et al., 1985; Steiner & Cameron, 1989). In the gonadectomized rat, the mean LH pulse frequency is three per hour (Tapper et al., 1972), whereas the gonadectomized rhesus monkey has an average pulse frequency of one pulse per hour (Dierschke et al., 1970). For this reason these LH pulses are often termed “circhoral”, meaning approximately hourly (Pohl & Knobil, 1982). In the agonadal or hypogonadal human, LH pulses occur once every one to two hours (Root et al., 1972; Yen et al., 1972; Santen and Bardin, 1973). In the female,

the frequency and amplitude vary greatly across the menstrual cycle due to both negative and positive feedback mechanisms of the ovarian steroid hormones estrogen and progesterone, ranging from low amplitude pulses once every one to two hours in the follicular phase, to high amplitude pulses once every eight to twelve hours in the middle of the luteal phase (Steiner & Cameron, 1989). Very slow pulses in the luteal phase result from the strong negative feedback of progesterone in the luteal phase (Crowley et al., 1985). In males, LH pulse frequency is once every 2-3 hours due to testosterone negative feedback (Veldhuis et al., 1989; Cameron et al., 1993; Schreihofer et al., 1996).

The menstrual cycle can be divided into the follicular phase (when the primary activity in the ovary is follicular development), ovulation and the luteal phase (when there is a functioning corpus luteum present). The follicular phase is typically the first 10-14 days of the cycle during which the growth of a dominant estrogen-secreting follicle(s) occurs that culminates in ovulation in which a mature oocyte is released in the reproductive tract for fertilization. FSH plays a large role in the recruitment and growth of fully mature ovarian (i.e., antral) follicles during the follicular phase of the menstrual cycle. However, it is the interaction of LH and FSH that is critical for ovarian steroidogenesis and normal menstrual cyclicity (Falck, 1959). During early follicular growth stages, granulosa cells within the follicle express receptors for FSH, while the theca cell layer of the follicle expresses LH receptors (Kobayashi et al., 1990). LH stimulates theca cells to produce androstenedione, which is then converted into estrogen by aromatase in the granulosa cells when stimulated by FSH (Barroso et al., 2001). In the late follicular phase, after exposure to both FSH and estrogen, granulosa cells acquire LH receptors and are thus able to produce their own androgenic precursor for estrogen,

leading to an exponential rise in estrogen in the very late follicular phase (Steiner and Cameron, 1989). Rising levels of estrogen produced by the dominant follicle have first a negative feedback effect that switches to a positive feedback effect once estrogen is sufficiently elevated for a critical duration at the level of the hypothalamus and pituitary (Speroff et al., 1999), leading to a large mid-cycle release of both LH and FSH, referred to as the LH/FSH surge (Figure 1.2). Thus, *both* gonadotropins are required for the follicle to produce an appropriate threshold concentration (i.e., 200 pg/mL in humans) and duration (i.e., 50 hours in humans) of estrogen secretion to induce positive feedback at the hypothalamus and pituitary, which is essential for triggering ovulation and subsequent fertility (Speroff et al., 1999). The surge controls the final development of the dominant follicle and triggers ovulation at the end of the follicular phase (Chappel and Howles, 1991; Shoham et al., 1993). After ovulation, a corpus luteum is formed from the reorganized cells of the dominant follicle and it releases both progesterone and estrogen. During the luteal phase estrogen and progesterone, along with inhibin, have negative feedback effects that serve to suppress gonadotropin secretion. As the cycle progresses, if the ovulated oocyte is not fertilized, the corpus luteum undergoes apoptosis and dies, and progesterone declines. Decreased progesterone support to the uterine endometrium results in a shedding of the uterine lining (Dobson et al., 2003). Decreasing progesterone also allows a more rapid frequency of pulsatile LH and FSH to occur, which stimulate the next wave of follicular development.

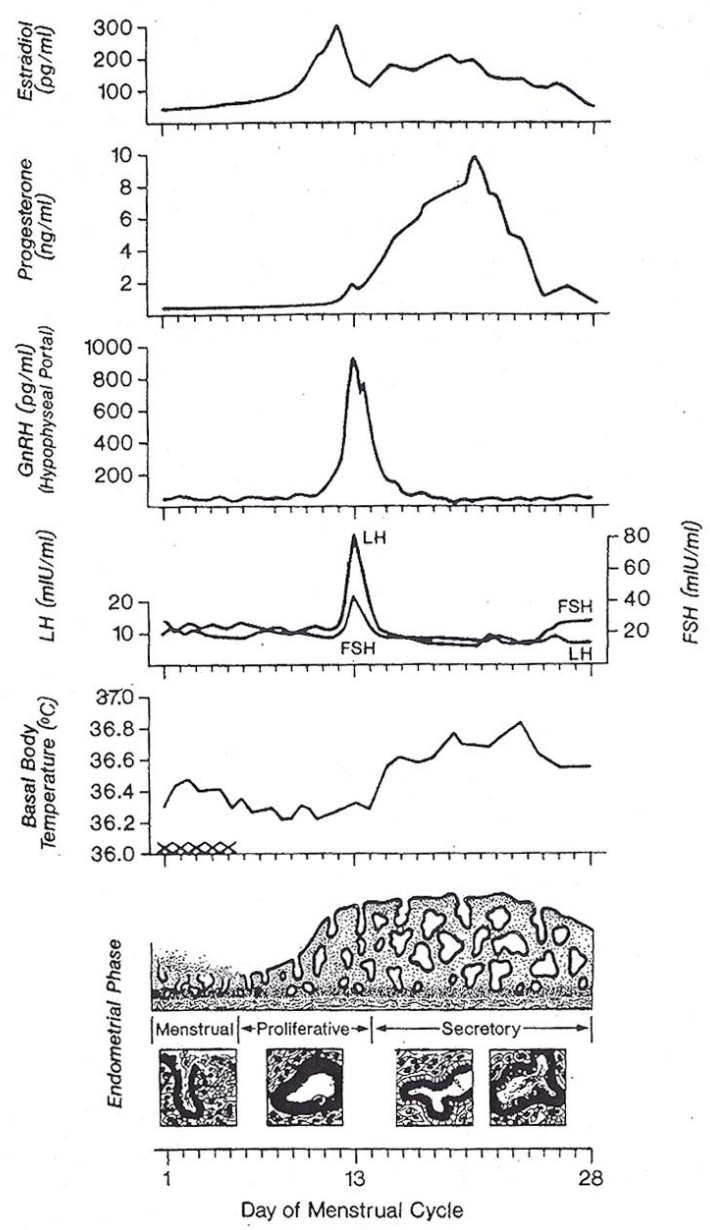


Figure 1.2 Changes in circulating levels of estrogen, progesterone, GnRH, LH and FSH, as well as body temperature and endometrial histology during the normal menstrual cycle (Steiner & Cameron, 1989).

1.2 The Role of Common Life Stresses in the Etiology of Reproductive Dysfunction

Females of many species can experience a suppression of reproductive function when exposed to stress, and this is particularly true in humans and other social primates in which the normal functioning of the reproductive axis can be influenced by exposure to even relatively mild psychosocial stresses (Cameron, 1997; Cameron, 2000; Marcus et al., 2001; Bomba et al., 2007). It has been well documented that stress-induced suppression of the reproductive axis can occur in response to chronic or severe stresses (Reifenstein, 1946; Bullen et al., 1985; Dubey et al., 1986; Giles & Berga, 1993; Berga et al., 1997; Resch et al., 2004). Many forms of chronic or severe stress can inhibit activity of the HPG axis, resulting in a suppression of circulating levels of reproductive hormones and eventually to infertility. Stressors that impact the functioning of the reproductive axis can be metabolic in nature, resulting from a decrease in circulating levels of available calories. Examples of such stressors include chronic undernutrition (Frisch, 1984; Dubey et al., 1986), chronic eating disorders (Warren et al., 1973; Resch et al., 2004), and regular participation in very strenuous exercise (Bullen et al., 1985; Williams et al., 2001a,b; Goodman & Warren, 2005). Psychosocial or psychogenic stressors have also been shown to suppress reproductive function, particularly in cases where the psychosocial stress is persistent, such as in mood disorders like chronic depression (Reifenstein, 1946; Giles & Berga, 1993; Berga et al., 1997). As with many fields, the study of the impact of stress on physiological functioning can be confounded by the somewhat ambiguous use of the term “stress”. For this reason, many in the field have adopted the term “allostasis” (McEwen, 2008) to refer to the active and ongoing process by which the body responds to daily challenges (i.e. “stressors”) and maintains

homeostasis. The term “allostatic load” refers to the physiological “cost” to the body from neuroendocrine and autonomic responses to these challenges (McEwen, 2008). In the clinical study of stress-induced reproductive dysfunction, the primarily concern has been with the accurate descriptions of the external stressors themselves rather than the maintenance of homeostasis. This is because it is well understood that the physiological reaction to severe stressors can result in a dysregulated allostatic state of suppressed reproductive function. However, it is also possible that stress-induced reproductive dysfunction could be induced by the allostatic load of everyday life stressors causing attrition to the normal physiological function of the body and brain, resulting in a new allostatic state, and that what is measured is the change to that new state, or allostasis. This dissertation will utilize the term “stress” or “allostatic load” throughout, while making a concerted effort to accurately and comprehensively define subtle differences in the perceived meaning of the term (i.e. psychosocial versus metabolic, chronic versus acute, etc.).

For both metabolic and psychosocial stresses, the reproductive axis is affected by chronic or severe stress at the level of the central nervous system, leading to a decrease in GnRH stimulation of the pituitary, a decrease in pituitary release of LH and FSH, and to a decrease in the activity of the ovaries, decreasing estrogen and progesterone and eventually resulting in anovulation and amenorrhea (Reame et al., 1985; Rasmussen et al., 1986; Berga & Girton, 1989; Foster et al., 1989; Loucks et al., 1989; Bronson & Manning, 1991; Wade & Schneider, 1992; Miller et al., 1993; Vigersky et al., 1997). More recently, it has been shown that even mild, acute stressors can impact reproductive function, such that there is a suppression of reproductive hormones and loss of normal

menstrual cyclicity (Rivier et al., 1986; Cameron & Nosbisch, 1991; Norman & Smith, 1992), especially when concomitant stresses are experienced (Williams et al., 2007). As with more severe stresses, if the mild stress is persistent there can be a suppression of fertility. Everyday life stresses, including stresses as mild as missing a single meal, can significantly impact the reproductive axis leading to a slowing of pulsatile luteinizing hormone (LH) secretion and a simultaneous suppression of circulating levels of testosterone in males and estrogen in females (Cagampang et al., 1990; Cameron & Nosbisch, 1991; Cameron et al., 1991; Olson et al., 1995). Acute forms of psychosocial stress, such as footshock and immobilization, can also lead to a rapid decrease in gonadotropin secretion in a variety of species (Rivier et al., 1986; Sapolsky & Krey, 1988; Norman & Smith, 1992). As with chronic or severe stress, the primary locus of dysfunction in the reproductive axis with exposure to acute stress appears to be at the level of the hypothalamus, in that administration of exogenous GnRH can stimulate normal gonadotropin secretion following acute stress (Rivier et al., 1986; Cameron & Nosbisch, 1991; Norman & Smith, 1992).

Clinically, one of the most common forms of stress-induced infertility is stress-induced amenorrhea, a condition previously known as functional hypothalamic amenorrhea or FHA. This reproductive disorder is a significant clinical issue, as it accounts for as much as 30% of the cases of female infertility (Reindollar et al., 1986). Stress-induced amenorrhea is defined as a sustained absence of normal menstrual cycles, despite healthy reproductive organs and the capability of showing normal physiologic functioning (Liu, 1990). For a number of years this disorder was considered a form of reproductive dysfunction in which psychological and/or behavioral responses to life

events led to a suppression of GnRH neuronal activity (Suh et al., 1988; Monzani et al., 1989; Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001). In psychological interviews, women with stress-induced amenorrhea report an increased degree of psychosocial stress in their lives compared to control populations, despite the fact that the numbers of divorces, family deaths, and other stressful life events were comparable to normally cycling women (Marcus et al., 2001; Bomba et al., 2007). However, a growing body of evidence has shown that some neuroendocrine aberrations associated with stress-induced amenorrhea are indicative of metabolic stress. Women diagnosed with this condition are of normal or slightly lower than normal body weight and they diet and exercise regularly (Berga & Girton, 1989). In fact, there is a high prevalence of aberrant eating patterns in this patient population, even though they do not meet the clinical criteria for eating disorders (Giles & Berga, 1993; Berga et al., 1997; Laughlin et al., 1998; Warren et al., 1999; Warren & Fried, 2001; Perkins et al., 2001). Furthermore, women with this disorder have low plasma levels of the thyroid hormone, triiodothyronine (T3), and elevated growth hormone (GH), which are both indicative of nutritional stress (Berga & Girton, 1989). Thus, stress-induced amenorrhea has been more recently attributed to a combined effect of mild psychosocial stress + metabolic stress, acting to suppress GnRH drive to the reproductive axis and normal reproductive function (Giles & Berga, 1993; Berga et al., 1997; Marcus et al., 2001). While there are numerous types of different stressors such as immune stress and illness (Barrell, 2007) or alcohol abuse and addiction (Mello, 1988) that might have a similar allostatic load on the reproductive axis, it appears from comprehensive clinical observation that the stress experienced in women with stress-induced amenorrhea is primarily psychosocial and

metabolic, and not other types of stress (Giles & Berga, 1993; Berga et al., 1997; Marcus et al., 2001).

1.3 Animal Models of Stress-Induced Reproductive Dysfunction

Animal models of stress-induced reproductive dysfunction have often employed methods designed to impact the HPG axis using various severe stressors in an attempt to maximize the physiological response to stress in all individuals equally. Studies in non-human primates have previously tested the effects of pharmacological or immunological stressors on activation of GnRH neuronal activity and menstrual cyclicity by administering endotoxin (Xiao et al., 1998; Xiao et al., 1999), corticotropin releasing hormone, or CRH, (Xiao and Ferin, 1988), and interleukin-1 (Feng et al., 1991; Ferin, 1995). While these studies have certainly improved our understanding of how various types of stress impact the activity of GnRH neurons, they do not provide adequate information about how the types of stressors involved in stress-induced amenorrhea (i.e. mild psychological stress, diet and exercise) actually lead to the suppression of reproductive function. Earlier studies attempted to model the stresses involved in stress-induced amenorrhea by studying the effects of psychosocial stress (Cameron et al., 1998), short-term fasting (Cameron & Nosbisch, 1991), and exercise training (Williams et al., 1991a; Williams et al., 1991b) on menstrual cyclicity, but these studies examined each form of stress separately.

Our laboratory has previously reported that the combination of mild psychosocial and metabolic stress is far more effective in the suppression of reproductive function than either stress alone (Williams et al., 2007). In that study, 3 groups of monkeys (n=9/group)

were exposed to either psychosocial stress alone (novel room), metabolic stress alone (diet and exercise), or a combination of mild psychosocial + metabolic stress. In the psychosocial stress alone and metabolic stress alone groups, only 1 out of 9 monkeys in each group exposed to a single stress alone became amenorrheic. However, in the group exposed to the mild combined stress of move + diet + exercise, 7 out of 9 monkeys lost menstrual cyclicity. This finding provided new insight into the possibility that there were individual differences in the sensitivity to everyday life stressors, and that there is synergism in the combination of psychosocial and metabolic stressors in the suppression of reproductive function.

As stress-induced amenorrhea is thought to be caused by mild psychosocial stress + metabolic stress acting synergistically to suppress GnRH drive to the reproductive axis (Giles & Berga, 1993; Berga et al., 1997; Marcus et al., 2001), it was important to use an animal model in which animals were exposed to multiple, simultaneous and clinically relevant stress exposures. The psychosocial and metabolic stresses used in the monkey model used in this dissertation were designed to replicate the stresses experienced by women seeking treatment for stress-induced amenorrhea (Berga & Girton, 1989; Berga et al., 1997) and therefore do not include models of other stressors such as immune or alcohol or drug exposure that have been used in other models of stress-induced reproductive dysfunction (Mello et al., 1997; Xiao et al., 1998; Xiao et al., 1999). Our laboratory has used female cynomolgus macaques (*Macaca fascicularis*) exposed to a mild psychosocial stress combined with a mild to moderate diet, with or without a moderate exercise regimen. Female cynomolgus monkeys are particularly useful for studies of individual differences in the sensitivity to stress-induced reproductive

dysfunction because they have year long monthly menstrual cycles like women (i.e., they are not seasonal breeders), and they live in complex social environments and are sensitive to psychosocial stresses.

The initial experimental paradigm utilized for these studies was a five-menstrual cycle design, as shown in Figure 1.3. Cycle 1 (CONTROL CYCLE 1) was a control cycle in which monkeys were allowed to have at least one normal menstrual cycle of 25-38 days in length (Williams et al., 2001a,b), during which time daily blood samples were collected to monitor ovarian hormone secretion. In cycle 2 (LEARN TO RUN), monkeys were trained to run on a treadmill, first by sitting, then standing and eventually walking on it. In cycle 3 (STRESS CYCLE 1) monkeys were moved on day 1 of the menstrual cycle to a single cage in a novel room, surrounded by unfamiliar monkeys, and maintained on a diet consisting of a 20% reduction in calories while initiating treadmill running (1 hr/ day, 5 days/week at 80% max speed). In cycle 4 (STRESS CYCLE 2), monkeys were again moved on day 1 of the cycle to a single cage in another novel room, while they were kept on the diet and exercise regimen. Cycle 5 (CONTROL CYCLE 2) was a recovery cycle in which monkeys were returned to their home environment, exercise training was terminated, and animals were returned to a full diet (Williams et al., 2007).

This animal model of stress-induced reproductive dysfunction is unique in its use of mild combined stresses (mild psychosocial stress + moderate diet and exercise) modeled after clinically reported levels of psychosocial and metabolic stresses experienced by women with stress-induced amenorrhea (Marcus et al., 2001). As such,

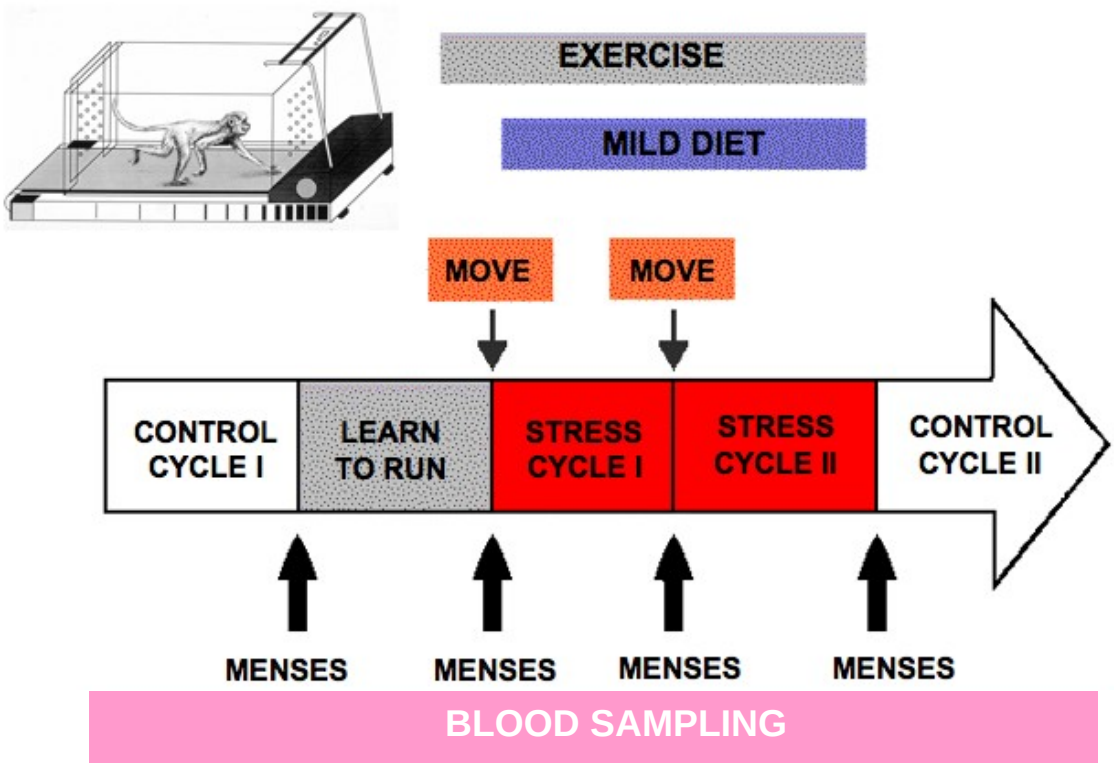


Figure 1.3 Schematic diagram of the experimental design implemented in the combined mild stress paradigm (adapted from Bethea et al., 2008).

the animal model used in this dissertation study provides excellent face validity to the human condition of stress-induced amenorrhea in women, both in the descriptions of external stressors, as well as in allostatic load and symptomatology (prolonged anovulatory cycles or amenorrhea, lower peak levels of estrogen and progesterone). However, there are some differences between the animal model and human condition. For example, a diagnosis of stress-induced amenorrhea requires women to be amenorrheic for at least 6 months, after ruling out other potential organic causes such as a pituitary adenoma. In contrast, this animal model only uses a two-menstrual cycle period of stress exposure, measuring if and how quickly a monkey loses reproductive function in response to mild combined stress. As such, this animal model may not necessarily be described in terms of measuring stress-induced amenorrhea *per se*, but rather the sensitivity to stress-induced reproductive dysfunction.

It remains unknown as to whether or not this animal model presents sound predictive validity, or if potential treatments tested in the model predict performance of treatments in the human condition. Our laboratory, in collaboration with others, has begun to characterize potential neural mechanisms underlying the etiology of stress-induced amenorrhea, including possible treatment approaches. In one such study, the Cameron laboratory administered citalopram, a selective serotonin reuptake inhibitor (SSRI) designed to inhibit synaptic reuptake and increase endogenous brain levels of serotonin, to monkeys with different sensitivity to stress. Interestingly, monkeys with increased sensitivity to stress-induced reproductive dysfunction showed increases in peak levels of estrogen and progesterone following treatment with citalopram, while those animals that were more resilient did not (Bethea et al., 2008). It remains to be seen

whether a similar treatment in the human condition would yield such promising results, thereby increasing the predictive validity of this animal model (as well as providing a potentially useful therapy for women with stress-induced amenorrhea). Lastly, the construct validity of this animal model is lacking in terms of neurobiological mechanisms, as the mechanisms in both the human condition of stress-induced amenorrhea and the experimental animal models are unknown at this time. However, similarity in the etiology of specific stressor types converging to suppress menstrual cyclicity in women and monkeys capable of reproductive function provide some theoretical rationale for the continued use of this model over others that may lack this clinical relevance.

Historically our laboratory has utilized this specific model of mild psychosocial stress + reduced diet + moderate exercise to categorize animals according to their reproductive axis response to these stressors, and has analyzed physiological and neurochemical features of stress-sensitive monkeys. As part of my dissertation studies, immunocytochemical approaches were used with brain tissue (see Chapter 4) from a group of animals exposed to this previously published animal model (Bethea et al., 2005a,b; Centeno et al., 2007a,b; Williams et al., 2007). However, physiological and pharmacological approaches (see Chapters 2 and 3) were used in a different group of chronically catheterized animals connected to a jacket/swivel/tether system for constant intravenous access (Cameron & Nosbisch, 1991). The chronic catheterization and use of a jacket/swivel/tether system in these animals made exercise training in this second group of monkeys difficult, as monkeys tended to develop skin sores under their arms and at their waists if they exercised while wearing a jacket. Therefore, the animal model used in

Chapters 2 and 3 does not include a Cycle 3 (LEARN TO RUN cycle), nor are the animals allowed to run on the treadmill at any time during the study. The only metabolic stressor used in these studies is a 20% reduction in available caloric intake. Our laboratory has previously published that the use of exercise training as a metabolic stressor is only suppressive to the reproductive axis because of the allostatic load of additional decreased caloric availability, as exercise-induced amenorrhea can be reversed in female macaques simply by increasing available caloric intake (Williams et al., 2001b). As such, it is possible, but unlikely, that these subtle differences in the animal model may present difficulty in interpretation and/or comparison to previously reported findings in which exercise was included in the animal model. Importantly, the exclusion of exercise training from the adapted mild combined stress model utilized in Chapters 2 and 3 of this dissertation did not affect the distribution or variability of individual differences in the sensitivity to stress-induced reproductive dysfunction observed in previously published reports using the original animal model (Bethea et al., 2005a,b; Centeno et al., 2007a,b; Williams et al., 2007).

1.4 The Concept of Stress-Sensitivity and Stress-Resilience

In a number of stress-related disease processes there are marked individual differences in the sensitivity to develop stress-induced physiological dysfunction, such that some individuals show marked physiological responses to stress and are more likely to develop stress-induced diseases ("stress-sensitive"), while others are more "stress-resilient", showing less of a physiological response to stressful stimuli and less

susceptibility to stress-induced diseases such as anxiety (Zvolensky & Leen-Feldner, 2005), depression (Cohen et al., 2005), hypertension (Treiber et al., 2003), gastrointestinal disorders (Levenstein et al., 2000), and suppressed immune function (Cohen & Hamrick, 2003). The stress sensitivity of an individual may be influenced by many factors, including genetics, prior stress exposure (particularly during early stages of development), and by the presence or absence of factors that provide protection from stress (McEwen, 2002).

Compared to other stress-induced disease states, the concept of sensitivity versus resilience to stress-induced reproductive dysfunction has received relatively little attention to date. A number of factors could influence the sensitivity of the reproductive axis to psychosocial stresses, including the intensity and duration of stress, the perception of stress, and amount of central drive to the reproductive axis prior to stress exposure (Cameron, 2000). Interestingly, individual differences in the sensitivity of the reproductive axis to stress have even been reported in an animal model utilizing a more profound immune stress challenges in non-human primates (Xiao et al., 1999).

Using the non-human primate model depicted in Figure 1.3, our laboratory has previously reported striking individual differences in the physiological responsiveness to mild combined stress (Cameron, 1997; Bethea et al., 2005a,b; Centeno et al., 2007a,b; Williams et al., 2007; Bethea et al. 2008). Approximately one-third of animals exposed to this paradigm continue to have normal, ovulatory menstrual cycles throughout the two cycles of combined mild stress exposure. These monkeys have been characterized as "highly stress-resilient" (HSR). Another third of the animals will ovulate and have one normal menstrual cycle upon initial exposure to mild combined stress, but with prolonged

exposure (STRESS CYCLE 2), will show a suppression of circulating levels of estrogen and progesterone and fail to mense within 38 days. These monkeys have been characterized as "medium stress-resilient" (MSR), because they show some menstrual cycle impairment only with repeated or prolonged exposure to stress and are therefore not as resilient to the effects of stress as are HSR monkeys. The final third of the animals will show an immediate suppression of estrogen and progesterone and fail to mense within 38 days of initial stress exposure (STRESS CYCLE 1). These monkeys have been characterized as "stress-sensitive" (SS), because they appear to be the most physiologically susceptible to the development of stress-induced reproductive dysfunction. The interesting finding that women with stress-induced amenorrhea tend to perceive their lives as more stressful than normal women (Marcus et al., 2001; Bomba et al., 2007) suggests that this disorder may occur primarily in women sensitive to everyday life stresses.

1.5 Characteristics of Stress-Sensitive Individuals

Using this above-described model of sensitivity to stress-induced amenorrhea, our laboratory has identified several stable physiological characteristics of stress-sensitive individuals. Monkeys that are most sensitive to stress-induced reproductive dysfunction have lower peak levels of estrogen in the follicular phase, and lower peak concentrations of progesterone in the luteal phase of a normal, non-stressed menstrual cycle (Bethea et al., 2008). This is a key feature of women with stress-induced amenorrhea, and provides face validity for our animal model (Berga & Girton, 1989). Though this is an important

finding, it does not identify the neuroanatomical origin of stress-induced impact upon the reproductive axis. As stress-induced amenorrhea is most notably a disorder in which stress affects the activity of the GnRH neurons in the hypothalamus, this finding of suppressed reproductive steroid hormone secretion in SS monkeys is supportive but not indicative of this stress-induced dysfunction in the reproductive axis at the level of the hypothalamus. Further experiments are necessary to determine that monkeys most sensitive to stress-induced reproductive dysfunction are impacted by psychosocial and metabolic stress at the level of the pituitary or above. As outlined later in this Introduction, studies in Chapter 3 of this dissertation begin to address these specific questions regarding the neuroanatomical origin of stress-induced reproductive dysfunction.

Our laboratory has been studying several possible neural mechanisms that might underlie sensitivity to stress-induced reproductive dysfunction. There are a number of neurotransmitter and/or neuropeptide systems which may, either directly and indirectly, mediate the response of the reproductive axis to stress, as there are multiple afferents to GnRH neurons in the hypothalamus which regulate the function of the HPG axis in the absence of stress (Dobson & Smith, 2000; Tilbrook et al., 2002; Dobson et al., 2003). It may also be important to keep in mind that SS monkeys have lower levels of estrogen and progesterone and low levels of these ovarian steroid hormones are associated with changes in activity of a variety of neurotransmitter systems, including serotonin (Ferin & Vande Wiele, 1984; Bethea et al., 1999; Roy et al., 1999; Saleh & Connell, 2003), that have afferent input into GnRH neurons. Our laboratory had shown that SS monkeys secrete less prolactin in response to a fenfluramine challenge (which causes central

release of endogenous serotonin), suggesting that SS monkeys produce and/or secrete less serotonin than HSR monkeys (Bethea et al., 2005a). The laboratory followed up this finding by examining the expression of several genes in the serotonin pathway and found that SS monkeys have significantly less gene expression for several important serotonin-related genes, including those involved in serotonergic cell development and differentiation (FEV1), the synthesis (tryptophan hydroxylase 2; TPH2), reuptake (serotonin transporter; SERT), receptor signaling (5HT1A), and degradation (monoamine oxidase A and B; MAO-A, MAO-B) of serotonin (Bethea et al., 2005b). The comprehensive downregulation of all these serotonin-related genes appears to be due at least in part to the fact that SS monkeys have fewer serotonin neurons in the dorsal raphe than monkeys with greater resiliency to stress (Bethea et al., 2005b), which might account for the simultaneous deficit in functionally opposing genes like TPH2 and MAO-A. Despite this finding, which might more accurately reflect increased apoptosis of serotonergic cells rather than downregulated serotonin-related gene expression *per se*, Bethea and colleagues (2009) have recently reported that even accounting for differences in the number of serotonin-positive cells in SS and HSR monkeys, serotonergic cells in the dorsal raphe nucleus of SS monkeys have lower gene expression of FEV1. This finding indicates that even the serotonergic neurons that survive apoptosis are not effective in the production of serotonin-related gene products, as compared to HSR monkeys. SS monkeys also have an upregulation of the serotonin receptors $5HT_{2a}$ and $5HT_{2c}$ in the hypothalamus, most likely occurring as a compensatory mechanism for suppressed secretion of serotonin in the brain (Centeno et al., 2007a). Interestingly, many of these serotonin receptors are located on GABAergic neurons in the medial basal

hypothalamus (Afione et al., 1990; Guptarakk et al., 2004). GABAergic neurons can provide strong inhibitory input to GnRH neurons leading to a decrease in central drive to the reproductive axis (Leranth et al., 1992; Dobson et al., 2003), and our laboratory has shown that SS monkeys have increased hypothalamic GAD67 (the rate-limiting enzyme in the synthesis of GABA) mRNA expression (Centeno et al., 2007a). Another neural system our laboratory has examined in monkeys differing in stress sensitivity is the CRH system. CRH neurons in the paraventricular nucleus (PVN) of the hypothalamus provide the central neuroendocrine drive to the hypothalamic-pituitary-adrenal (HPA) axis, in addition to maintaining diverse connectivity as a neurotransmitter (McEwen, 2008). This is particularly interesting as CRH has also been shown to provide inhibitory input to GnRH neurons (Petraglia et al., 1987; Williams et al., 1990; Feng et al., 1991; Briski et al., 1995; Rivest & Rivier, 1995) and could play a role in causing the suppression of reproductive function in stress-sensitive individuals.

Using this non-human primate model of sensitivity to stress-induced reproductive dysfunction, Centeno and colleagues (2007b) have shown that stress-sensitive monkeys have elevated CRH mRNA expression in the most caudal regions of the PVN, as well as in the amygdala. This area of the PVN consists primarily of parvocellular neurons which project to a variety of limbic and autonomic areas, including the bed nucleus of the stria terminalis (BNST) and central amygdala (Walker et al., 2001; Crane et al., 2003), the locus coeruleus (Chowdhury et al., 2000; Makino et al., 2002; Reyes et al., 2005), and the medulla and spinal cord (Sawchenko & Swanson, 1982; Luiten et al., 1985; Puder & Papka, 2001). Therefore, as CRH neurons regulate both the HPA axis via neuroendocrine mechanisms as well as stress-related processes in extra-hypothalamic brain regions, the

increased CRH expression seen in the PVN of stress-sensitive animals may or may not be related to an increased activation of the HPA axis.

1.6 The Role of the Hypothalamic-Pituitary-Adrenal (HPA) Axis in Stress-Induced Reproductive Dysfunction

CRH has been shown to directly suppress GnRH neuronal activity in several species (Petraglia et al., 1987; Williams et al., 1990; Feng et al., 1991; Briski et al., 1995; Rivest & Rivier, 1995). The CRH system is a complex network of neurons that, in addition to having neuroendocrine action at the median eminence to regulate the HPA axis, also acts as a neurotransmitter, projecting to numerous sites in the brain to regulate various stress-associated behaviors and physiological processes (Koob, 1999). CRH is thought to have not only a direct role in mediating stress-related processes in the brain, but also extensive interaction with other neurotransmitter systems to integrate various autonomic and behavioral components of the stress response (Dunn & Berridge, 1990; Valentino et al., 1993). Importantly, release of CRH from the PVN of the hypothalamus provides the central drive to the HPA axis, stimulating downstream release of ACTH from the pituitary and cortisol from the adrenal glands. Elevated secretion of both ACTH and cortisol has been documented following exposure to a variety of stressors, including psychosocial (Smith & French, 1997; Fuchs & Flugge, 2002; Kudielka et al., 2004) and metabolic stressors (Rosmond et al., 1998; Leal-Cerro et al., 2003). Stress-induced activation of the HPA axis is thought to reflect increased secretion of hypothalamic CRH (McEwen, 2008). Like CRH, glucocorticoids can also suppress activity of the

reproductive axis if the elevated circulating levels are *chronic* in nature. In rodents, chronic corticosterone treatment can suppress circulating levels of gonadotropin and gonadal steroid hormones (Baldwin & Sawyer, 1974; Baldwin, 1979; Ringstrom & Schwartz, 1985), prevent ovulation (Baldwin, 1979; Smith et al., 1971) and decrease the sensitivity of the pituitary to GnRH stimulation (Baldwin & Sawyer, 1974; Baldwin, 1979; Ringstrom & Schwartz, 1985; Kamel & Kubajak, 1987). Likewise, in monkeys and humans, chronic elevation of cortisol can also suppress gonadotropin and gonadal steroid hormone secretion (Boccuzzi et al., 1975; Luton et al., 1977; Plant et al., 1983; Dubey & Plant, 1985; Sapolsky, 1985).

Interestingly, cortisol, typically used as a marker of HPA axis activation, has been reported as elevated in women with stress-induced amenorrhea (Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001), although this has not been a consistent finding (Couzinet et al., 1999). The timing, with regard to time of day, of the elevation in cortisol levels associated with stress-induced amenorrhea is controversial, as some investigators have reported elevated cortisol in women with this disorder during the nighttime hours (Berga et al., 1997), whereas other investigators have reported elevated cortisol during the daytime (Suh et al., 1988). Cortisol is also elevated in the cerebrospinal fluid of women with stress-induced amenorrhea (Brundu et al., 2006). While there is indeed some evidence that activity of the HPA axis is elevated in women with stress-induced amenorrhea, because this patient population is more sensitive to everyday life stress (Marcus et al., 2001; Bomba et al., 2007), it is unclear whether these findings reflect elevated *baseline* secretion or an elevated *response to stress*. It is also unknown whether women with this disorder fail to show normal sensitivity to negative

feedback regulation by cortisol, as this specific aspect of HPA axis activity has received little attention. One or more of these mechanisms may account for increased activity of the HPA axis in women with stress-induced amenorrhea. Elevated activity of the HPA axis has been demonstrated as a neural mechanism for some psychosocial (Smith & French, 1997; Fuchs & Flugge, 2002; Kudielka et al., 2004) and metabolic (Rosmond et al., 1998; Leal-Cerro et al., 2003) stresses, both of which are present in clinical stress-induced amenorrhea and are utilized in this non-human primate model.

The overall hypothesis of this dissertation is that increased activity of the HPA axis is an underlying causal mechanism mediating the sensitivity to stress-induced reproductive dysfunction in female monkeys, as a model for sensitivity to stress-induced amenorrhea in women. Studies in Chapter 2 used physiological techniques in chronically catheterized monkeys to test the hypothesis that elevated HPA axis activation is associated with sensitivity to stress-induced reproductive dysfunction. Specifically, in Chapter 2 I characterized activity of the HPA axis in six different ways to ask whether stress-sensitive monkeys had elevated ACTH and cortisol as compared to more stress-resilient monkeys. In Chapter 3, I utilized pharmacological techniques to test the hypothesis that elevated HPA axis activity was not only associated with, but causal to, the sensitivity to stress-induced reproductive dysfunction. In these studies, I used a specific CRH receptor antagonist to suppress activation of the HPA axis and test whether or not this inactivation resulted in a prevention of stress-induced reproductive dysfunction. Additionally, physiological studies in Chapter 3 tested the hypothesis that stress acts centrally at the level of the pituitary or above to suppress reproductive function, by collecting baseline LH pulse frequency measurements in stress-sensitive and stress-

resilient monkeys in a non-stressed, control menstrual cycle. I hypothesized that stress-sensitive monkeys, that have lower peak levels of estrogen and progesterone in non-stressed menstrual cycles, would also have slower LH pulse frequencies than more stress-resilient individuals. In Chapter 4, I used immunocytochemical techniques to examine a possible interaction between the CRH neurotransmitter system and the serotonin system, another candidate neurotransmitter that might contribute mechanistically to the etiology of stress-induced amenorrhea. This study was conducted using brain tissue from a different group of monkeys euthanized prior to the beginning of my dissertation work. Based on the findings from Chapters 2 and 3, I tested the hypothesis that stress-sensitive monkeys would have increased CRH neuronal input to the dorsal raphe nucleus, as CRH is known to be inhibitory to the synthesis and release of serotonin (Price et al., 1998; Kirby et al., 2000; Price & Lucki, 2001), and might partially explain our previous findings that stress-sensitive monkeys have downregulated serotonin gene expression and fewer serotonin cells in this brain region (Bethea et al., 2005a,b; Centeno et al., 2007a). Taken together, the studies in this dissertation examine neuroendocrine function, pharmacological intervention, and central neural pathways as a means to determine whether differences in the activity of the HPA axis might underlie individual differences in the sensitivity of the reproductive axis to stress, and to further characterize neurophysiological and behavioral features of stress-sensitive individuals. Figure 1.4 illustrates the timeline of experiments conducted in this dissertation.

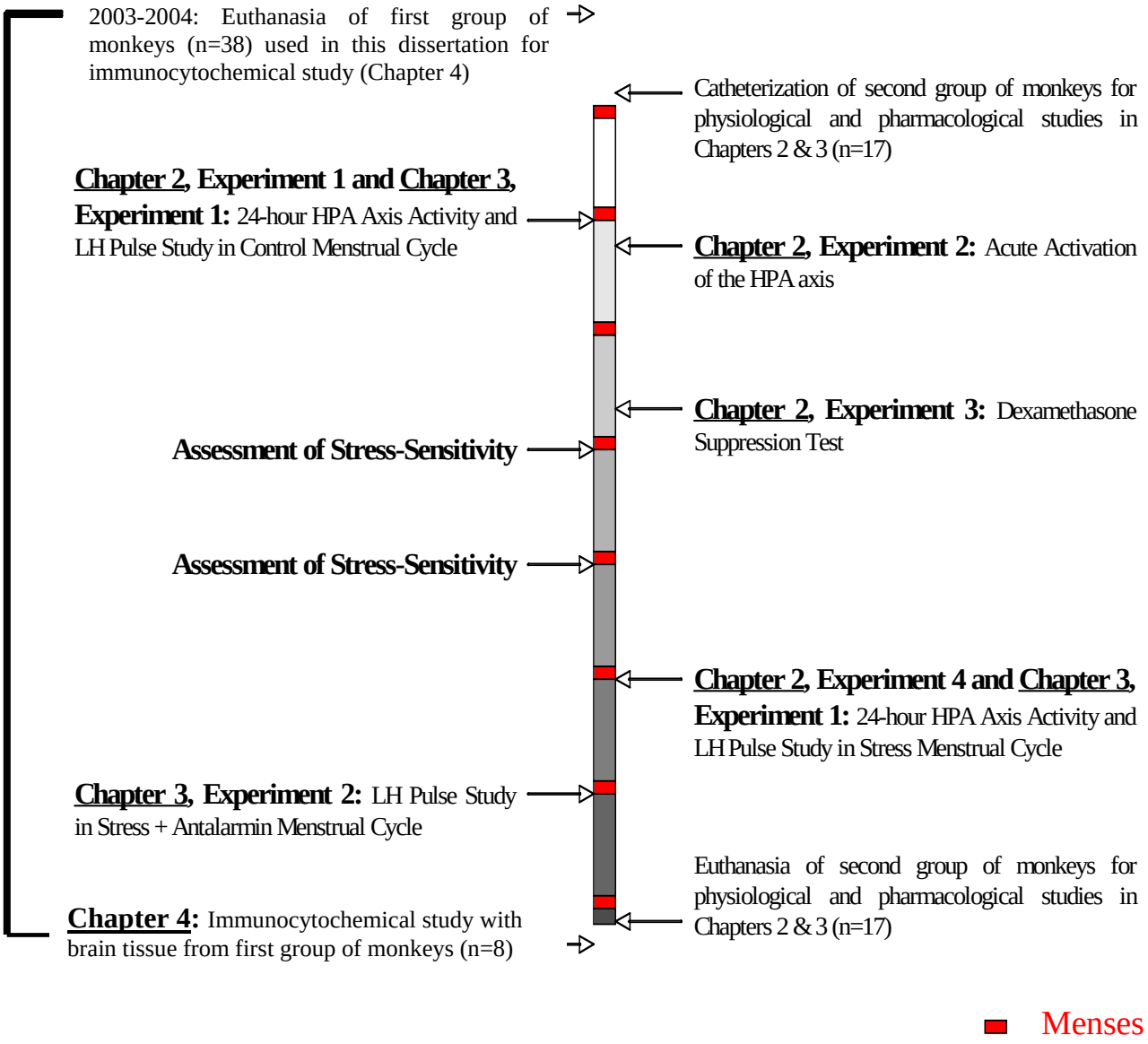


Figure 1.4 Schematic diagram of the experimental timeline of studies detailed in this dissertation.

Chapter 2

SENSITIVITY TO STRESS-INDUCED REPRODUCTIVE DYSFUNCTION IS NOT ASSOCIATED WITH ELEVATED BASELINE ACTIVITY OF THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

2.1 INTRODUCTION

Stress-induced amenorrhea is a common, non-organic form of stress-induced infertility (Liu, 1990), thought to be caused by a combined effect of mild psychosocial stress + metabolic stress, acting to inhibit GnRH drive to the reproductive axis and suppress normal reproductive function (Giles & Berga, 1993; Berga et al., 1997; Marcus et al., 2001). Elevation of the hypothalamic-pituitary-adrenal (HPA) axis has been suggested as a potential neural mechanism underlying the etiology of stress-induced amenorrhea, as cortisol has been shown to be elevated in this patient population (Suh et al., 1988; Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001; Lindahl et al., 2007), although this has not been reported in all studies of this disorder (Couzinet et al., 1999). There are several different mechanisms which may underlie elevated activity of the HPA axis in stress-induced amenorrhea, including elevated basal activity of the HPA axis, elevated response of the HPA axis to stress, and/or decreased sensitivity of the central neural drive to the HPA axis to glucocorticoid negative feedback.

Cortisol secretion in women with stress-induced amenorrhea has been studied over the 24-hour day and some studies show elevations in cortisol compared to women with normal menstrual cycles (Suh et al., 1988; Biller et al., 1990; Berga et al., 1997;

Lindahl et al., 2007). However, there is controversy regarding the timing of the elevation in cortisol during the diurnal cycle, as some investigators have reported elevated cortisol levels in women with stress-induced amenorrhea only during the nighttime hours (Biller et al., 1990; Berga et al., 1997), only during the daytime hours (Suh et al., 1988), or both during the day and night (Lindahl et al., 2007). It may also be important that these studies have been performed in a clinical setting and therefore may not be representative of true baseline activity of the HPA axis. While studies examining the response of the HPA axis to acute psychosocial stress in women with stress-induced amenorrhea are lacking, several reports have indicated that women with this disorder have adrenocorticotropic hormone (ACTH) responses to acute corticotropin-releasing hormone (CRH) challenge that are actually lower than in normal women (Meczekalski et al., 2000; Kondoh et al., 2001), which would be indicative of more chronic activation of the HPA axis and chronic cortisol negative feedback. However, there have also been reports that CRH responsiveness is no different (Biller et al., 1990) than in healthy control women. Sensitivity to glucocorticoid negative feedback has been least studied in this patient population. However, Lindahl and colleagues (2007) reported that women with stress-induced amenorrhea do not have decreased sensitivity to glucocorticoid negative feedback, as measured by a dexamethasone suppression test (Carroll et al., 1981). Thus overall, there is some evidence that there is increased activation of the HPA axis in women with stress-induced amenorrhea compared to women who display normal menstrual cycles, but the evidence is weak and is certainly not strong enough to indicate that increased activation of the HPA axis plays a causal role leading to stress-induced amenorrhea.

Our laboratory has developed a non-human primate model of sensitivity to stress-induced amenorrhea in which female cynomolgus macaques, that have 28-day menstrual cycles like human women, are exposed to a combination of mild psychosocial stress plus a mild metabolic stress of reduced diet with or without exercise (Cameron, 1997; Cameron, 2000; Bethea et al., 2005a,b; Centeno et al., 2007a,b,c; Williams et al., 2007; Bethea et al., 2008). Using this model, previous work has shown that monkeys differ in their sensitivity to stress-induced reproductive dysfunction, such that some monkeys readily lose reproductive function with exposure to mild combined stress (i.e. are "stress-sensitive"), while others are more stress-resilient. In the studies presented in this chapter, I tested the hypothesis that female cynomolgus monkeys showing increased sensitivity to stress-induced reproductive dysfunction, like women with stress-induced amenorrhea, would have greater activation of the HPA axis by one or more of the specific mechanisms discussed above.

2.2 MATERIALS AND METHODS

Animals

Seventeen adult female cynomolgus monkeys (*Macaca fascicularis*), 7-10 years of age, were used in these studies. Monkeys were housed in individual stainless steel cages in a temperature-controlled room (23±2°C) with lights on for 12 h per day (0700h to 1900h). Animals were fed two meals a day consisting of four high-protein monkey chow biscuits (no. 5047, jumbo biscuits; Ralston Purina Co., St. Louis, MO) at 0930h and 1530h, and a supplement of one-quarter a piece of fresh fruit was provided with each afternoon meal. Animals had their vaginal area swabbed daily with a cotton-tipped swab

to detect menstrual bleeding. The first day of menses was designated Day 1 of a menstrual cycle. Food intake was measured at each meal and weight was measured weekly. All protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center.

Catheterization

Each monkey had a chronic indwelling venous catheter placed in a subclavian vein, using previously described methods (Cameron & Nosbisch, 1991). Catheterization surgeries were performed under isoflurane anesthesia (VedCo, St. Joseph, MO). At surgery, the tip of the catheter was positioned in the right atrium of the heart and the free end of the catheter was routed subcutaneously to the back and threaded through a small cutaneous incision between the scapulae. To protect the catheter line, each animal wore a fitted nylon jacket that was connected to a 36-inch flexible metal tether that attached to a swivel mounted on the top of the monkey's cage. Silastic tubing extended from the top of the swivel through a hole in the wall to a sampling syringe and stopcock, attached to a saline infusion system, in the adjacent room. This catheter system allowed for blood sampling and intravenous infusions without sedating or disturbing the animal, while allowing the monkey free range of motion within the cage. Catheter lines were kept patent with a constant infusion of physiological saline (Baxter Healthcare, Deerfield, IL) containing heparin sodium (4 IU/mL) at a rate of approximately 100 mL/day. Weekly inspections of catheter systems and replacement of a sterile dressing covering the catheter exit site were performed under Ketaset (ketamine hydrochloride, 10 mg/kg i.v.) to keep the exit site aseptic and prevent infection. Ketamine was never administered within 24

hours preceding an experimental procedure. Following catheterization surgery, animals were allowed enough of an adaptation period to the jacket/swivel/tether system in order to resume normal menstrual cyclicity before any experimental procedure was performed.

Study Design

Prior to the studies presented in this chapter, monkeys had been maintained in a stable social environment on their standard diet and had shown at least one normal menstrual cycle (CONTROL CYCLE I), determined by a peak luteal phase progesterone of 2 ng/mL or higher (indicating ovulation) and normal cycle length (25-38 days; Williams et al., 2001a,b). Figure 2.1 depicts the experimental timeline of the studies in this chapter. Monkeys were tested in two cohorts, the first (n=7) being tested in all studies presented in Chapters 2 and 3 between 2005 and 2007, and the second (n=10) being tested between 2007 and 2008. Before each experiment, each monkey had to show at least one normal menstrual cycle before progressing on to the next planned experiment. For example, if the monkey had a normal menstrual cycle following Experiments 1 and 2, Experiment 3 was conducted in the luteal phase of the following cycle. If the animal failed to exhibit a normal cycle following Experiments 1 and 2, Experiment 3 was postponed until after the animal showed a normal menstrual cycle. Following completion of Experiments 1-3, the animals underwent characterization for stress-sensitivity of the reproductive axis; the mild combined stress paradigm lasting for a two-cycle duration (STRESS CYCLE I & II) as described below. Once each monkey exhibited at least one normal menstrual cycle following exposure to the mild combined stress paradigm (CONTROL CYCLE II), Experiment 4 was conducted on day 1 of the next cycle.

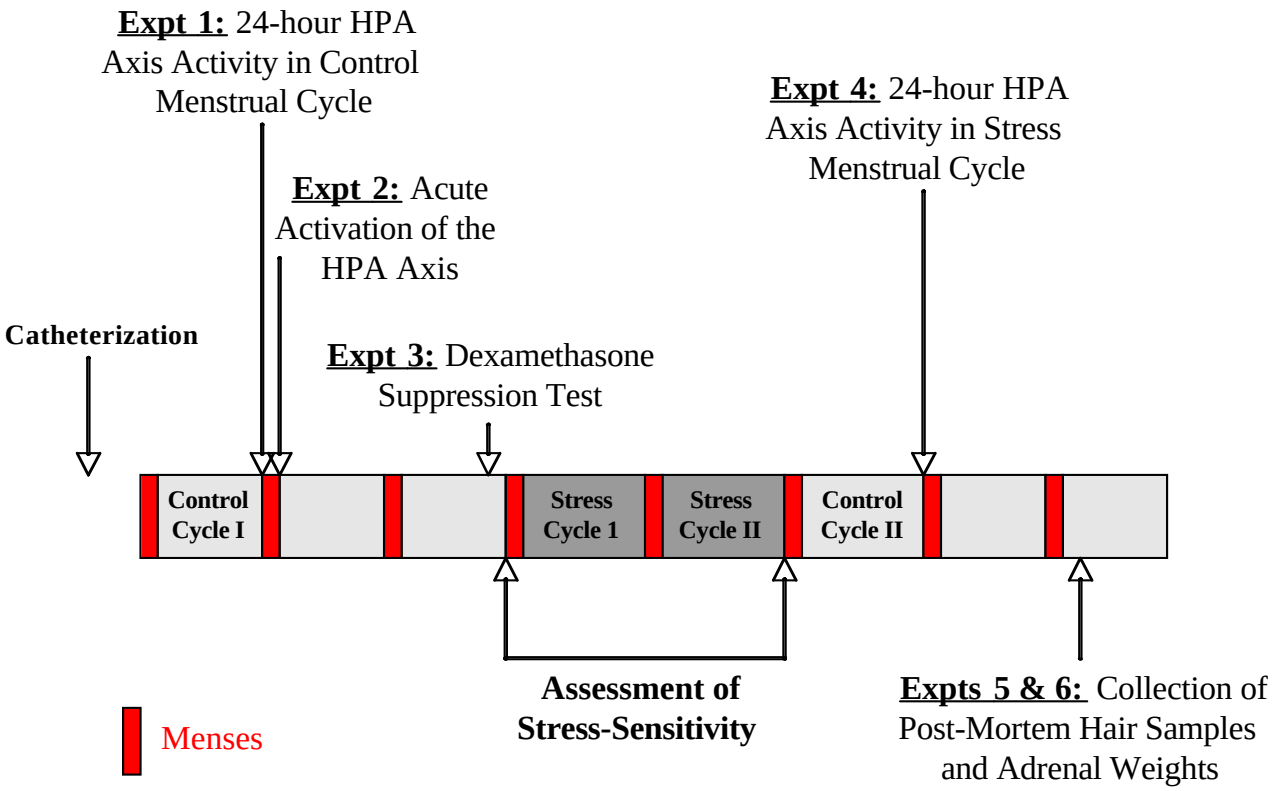


Figure 2.1 Schematic diagram of the experimental timeline of studies detailed in Chapter 2.

Data for Experiments 5 and 6 were collected at the time the animals were euthanized, after completion of all physiological and pharmacological studies presented in Chapters 2 and 3.

Characterization of Stress-Sensitivity of the Reproductive Axis

Monkeys were studied for two menstrual cycles to evaluate the sensitivity of the reproductive axis to stress. Because this paradigm involved exposure to psychosocial and metabolic stress, this characterization was performed after completion of Experiments 1, 2, and 3 (detailed below), as these three experiments were measures of baseline HPA axis activity. Following completion of Experiments 1-3, animals were exposed to a combined psychosocial and metabolic stress and the response of the reproductive axis to this stress was determined in order to categorize each monkey as highly stress-resilient (HSR), medium stress-resilient (MSR) or stress-sensitive (SS). On Day 1 of the first "stress" menstrual cycle, animals were moved to a new cage in a novel housing room surrounded by unfamiliar monkeys and they were placed on a moderate diet that had 20% fewer calories than their average daily intake in the preceding cycle. Blood samples (0.6 mL/sample) were taken every other day using sterile techniques to measure reproductive steroid hormone levels. Monkeys that mensed within 25-38 days subsequent to the initiation of stress were moved for a second stress cycle and remained on reduced calorie intake diet (Williams et al., 2001a,b). As established by previous studies (Williams et al., 2007), animals that showed a suppression of reproductive function in the first "stress" cycle and did not show menses within 38 days were categorized as "stress-sensitive" (SS). Animals that did not show suppression of reproductive function in response to both

stress cycles 1 and 2 were categorized as “highly stress-resilient” (HSR). Some animals had a normal cycle for the first stress cycle but suppressed normal menstrual cyclicity in the second stress cycle. These animals were categorized as “medium stress-resilient” (MSR).

Experimental Protocols

Activity of the HPA axis was assessed in six ways. Baseline activity of the HPA axis over a normal day was evaluated by measuring plasma ACTH and cortisol levels every two hours for 24 hours. Responsiveness of the HPA axis to acute stress in a control, non-stressed cycle was evaluated by measuring ACTH and cortisol responsiveness to an acute, standardized psychological stress. The strength of the central neural drive to the HPA axis was evaluated by measuring the ability of dexamethasone to inhibit HPA axis activity. Diurnal activity of the HPA axis was again measured in response to a mild combined psychosocial + metabolic stress used to categorize sensitivity of the reproductive axis to stress, measuring ACTH and cortisol every 2 hours in a menstrual cycle during stress exposure. Concentration of cortisol in a sample of hair and adrenal weights, collected post-mortem, were also measured as integrative measures of HPA axis activity. Monkeys were sacrificed after three days of acute psychosocial + metabolic stress exposure.

Experiment 1. Characterization of Baseline Diurnal ACTH and Cortisol Secretion

To measure baseline activity of the HPA axis, plasma ACTH and cortisol levels were measured over a normal 24-hour period in the early follicular phase of the menstrual

cycle, between Day 2 and Day 4 of the cycle. Blood samples (0.4 mL/sample) for ACTH and cortisol were collected into separate syringes via the remote catheter system at 2-hour intervals from 0800 h until 1000 h the following day. Samples to be assayed for cortisol were collected into heparinized syringes and placed in an empty sterile plastic centrifuge tube on ice, while blood samples to be assayed for ACTH were collected into non-heparinized syringes and placed in a sterile plastic centrifuge tube containing 20uL of 28.8 mg/mL EDTA on ice. Both samples were immediately centrifuged at 3000 rpm for 15 minutes at 4°C. Plasma samples were pipetted into plastic O-ring storage vials (for cortisol the vials contained 20 µL of a solution composed of equal volumes of 30% sodium citrate and 1,000 IU/mL sodium heparin to prevent clotting of plasma proteins; for ACTH the vials contained 10uL 500 KIU/mL aprotnin to prevent peptide degradation). Samples were stored at -20°C until assays were performed. Red blood cells from cortisol samples only (not containing EDTA) were resuspended in sterile heparinized saline and reinfused through the catheter system. Hematocrit was checked at intervals during the study and remained in the normal physiological range throughout the sampling period.

Experiment 2. HPA Axis Activation to Acute Stress

To characterize the intensity and duration of HPA axis activation in response to a standardized acute stressor, leather “catch gloves” were presented to each monkey in a standardized protocol (Helmreich et al., 1993; Rogers et al., 1999). These gloves are typically used to hand-catch unanesthetized monkeys, and our lab has previously reported that this paradigm is a significant psychological stressor, inducing anxiety-related

behaviors such as open mouth threats or vocalizations, and elevating plasma ACTH and cortisol (Helmreich et al., 1993; Rogers et al., 1999). This study was performed on the day after completion of Experiment 1. As plasma concentrations of ACTH and cortisol fluctuate over a normal day, Experiment 2 was always conducted at 1600 h, when ACTH and cortisol levels were lower than in the morning hours, in order to be able to observe stress-induced increases in ACTH and cortisol, and to limit variability due to diurnal fluctuations. A baseline blood sample for ACTH and cortisol (0.4 mL/sample) was collected via the remote catheter system at 1600 h. Following the collection of a baseline sample, a person unfamiliar to the monkey (a female who was not the animal's primary caretaker, wearing a red lab jacket) entered the room and stood next to the monkey's cage, presenting the leather "catch gloves" to the monkey at 1615, 1630, and 1645 h, for a two minute duration at each entry. The person with the catch gloves on manipulated the lock on the cage, touched the tops and sides of the cage, and pretended to initiate capture of the animal, but did not open the cage. Following the last entry of the person wearing catch gloves, the door to the room was shut for the evening, according to normal nighttime procedures. Blood samples for ACTH and cortisol (0.4 mL/sample) were then collected at 1700, 1800, and 1900 h, using the same procedures detailed above.

Experiment 3. Dexamethasone Suppression of the HPA Axis

To evaluate individual differences in the sensitivity of the HPA axis to glucocorticoid negative feedback, animals were given a dexamethasone suppression test in the luteal phase, on Day 17 of the subsequent menstrual cycle. This experiment was conducted in the luteal phase of the menstrual cycle in order to perform the experiment

twice in a small pilot group of animals, when first developing the dexamethasone assay (see section on Hormone Assays), as the luteal phase is a longer period of time in which endogenous hormonal milieu is more consistent than in the follicular phase. A baseline blood sample for cortisol (0.6mL/sample) was collected at 0800h, and the synthetic glucocorticoid dexamethasone sodium phosphate (150 µg/kg, Henry Schein, Melville, NY) was administered to each monkey at 1700h that evening (Carroll et al., 1981). Blood samples (0.6 mL/sample) for cortisol and dexamethasone were collected prior to infusion and at 5, 15, 30, and 60 min and at 2h, 6h and 15h after dexamethasone administration to calculate percent suppression of cortisol as well as individual clearance rates of dexamethasone in each animal, using the procedures detailed above.

Experiment 4. Characterization of Diurnal ACTH and Cortisol Secretion Following Exposure to Mild Combined Stress

To measure 24-hour activity of the HPA axis in response to mild psychosocial + metabolic stress, plasma ACTH and cortisol levels were measured over a 24-hour period on Day 1 of a menstrual cycle with acute exposure to mild psychosocial + diet stress. On Day 1 of a menstrual cycle, the animal was moved to a single cage in a novel room at 0930 h and placed on a 20% reduced calorie diet. Blood samples (0.4 mL/sample) for ACTH and cortisol were collected into separate syringes via the remote catheter system at 2-hour intervals from 1000 h until 1000 h the following day, using procedures identical to those in Experiment 1. Due to initial concerns regarding the volume of blood collected from these monkeys over the entire experimental period, some animals were only sampled for ACTH and cortisol every 4 hours. Because direct comparisons between a

control menstrual cycle and a stressed cycle were not possible at every time point, mean daytime (1000 h – 1800 h) and nighttime (2200 h – 0600 h) ACTH and cortisol values were calculated and compared. Red blood cells from cortisol samples only (not containing EDTA) were resuspended in sterile heparinized saline and reinfused through the catheter system. Hematocrit was checked at intervals during the study and remained in the normal physiological range throughout the sampling period. Upon completion of the 24-hour experimental period, the animal remained in the novel room and was returned to a full diet to resume normal menstrual cycles.

Experiment 5. Measurement of Hair Cortisol

Monkeys were euthanized at the end of their experimental protocols according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. After completion of all physiological experiments, each animal was moved on the first day of their final menstrual cycle to a novel room and placed on a 20% reduced calorie diet according to the mild combined stress paradigm detailed below. On the fourth day of their cycle, each animal was sedated with ketamine in the home cage, transported to the necropsy suite and given an overdose of sodium pentobarbital (25 mg/kg, i.v.) for euthanasia. Following administration of pentobarbital, hair was carefully shaved from the posterior vertex region of the neck, between the cisterna magna and scapular bones, before continuing with perfusion. Procedures for collection and treatment of hair samples were followed as established by Davenport et al. (2006). Briefly, approximately 250 mg of hair was washed twice with isopropanol by gentle inversion, dried for 6 days, and ground to a fine powder with a ball mill grinder.

Methanol (1.0 mL) was added to approximately 50 mg of hair powder and samples were rotated gently overnight to extract cortisol from the powder. The samples were then centrifuged for 30 seconds and 600 μL of the methanol extract was transferred to a new tube. After evaporation of the solvent, the dried extracts were reconstituted with 400 μL assay buffer and then analyzed in duplicate by enzyme immunoassay following the manufacturer's instructions (Salimetrics, State College, PA). Resulting values were converted from μg/dL to pg/mg for analysis.

Experiment 6. Adrenal Weights

Following administration of pentobarbital and collection of hair, monkeys were exsanguinated by severance of the descending aorta. The left ventricle of the heart was cannulated and the head of each animal was first perfused with 1 liter of saline. Left and right adrenal glands, along with other tissues, were quickly removed during the saline infusion period, weighed, flash frozen on isopentane and stored at -20 for future studies. Immediately following infusion of 1 liter of saline, perfusion continued with 7 liters of 4% paraformaldehyde in 3.8% borate, pH 9.5, after which the brain was removed and processed for other studies.

Hormone Assays

All plasma assays were performed by the Endocrine Services Laboratory (ESL) of Oregon National Primate Research Center. Plasma cortisol and ACTH, as well as estrogen and progesterone, were measured using an Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL), an automatic clinical platform using polystyrene beads

coated with hormone-specific antibodies as the solid phase and chemiluminescent quantifications. The ONPRC assay core has validated the use of the Immulite 2000 for several hormones in the rhesus monkey including cortisol, ACTH, estrogen and progesterone. Validation and comparison between the Immulite ACTH assay and the Nichols Advantage ACTH assay has been published (Vogeser et al., 2000). Both assays yield similar results for measuring ACTH in human samples, including linearity (r^2 > 0.99), range (10 – 1250 pg/ml for Immulite), and consistency in sample values (Pearson's r = 0.98). Validation of the Immulite cortisol assay was performed by direct comparison of monkey serum samples analyzed coordinately by the Immulite 2000 and a Roche Elecsys 2010 analyzer (also a chemiluminescence-based clinical platform by F. Hoffmann-La Roche Ltd, Basel Switzerland) that had been previously validated for monkey cortisol measurements (Bethea et al., 2005a). The comparison involved 109 monkey samples with cortisol levels ranging from 20 - 550 ng/ml. The coefficient of correlation between the two platforms was 0.95. Sequential samples taken over 24 hours showed a distinct elevation in cortisol levels in the morning measured by both assay platforms.

The validation for estrogen and progesterone included a direct comparison of monkey serum samples analyzed coordinately by an Immulite 2000 and a Roche Elecsys 2010 analyzer that had been validated for monkey steroid measurements (Jensen et al., 2008). The comparison involved 105 monkey samples with estradiol levels ranging from 10 - 400 pg/mL. The coefficient of correlation between the two platforms was 0.93. The progesterone values in 62 monkey samples ranged from 0.2 - 9 ng/mL; the coefficient of correlation between the two platforms was 0.95. The sensitivity of estrogen assays by the

Immulite 2000 was 20 pg/mL, and for progesterone was 0.2 ng/mL. All quality control samples and validations provided by the company were analyzed each time before hormonal measurements in samples were made. As with many validated clinical platforms, the Immulite 2000 runs three QC serum pools daily and as such, no specific intra-assay QC data is available. The inter-assay coefficient of variation, reflecting variability in daily QC results over a period of 1.5 years in which these assays were run, was as follows: ACTH 7.7%; cortisol 8.1%; estradiol 8.5%; progesterone 9.4%.

For DEX clearance calculations, plasma dexamethasone concentration was assayed with an ELISA assay from Neogen Corp, Lexington KY. This assay was originally a forensic assay and thus was designed to provide a simple positive or negative evaluation of whether dexamethasone was present in the biological fluid of interest. The ESL core prepared DEX standards from crystalline steroid (Steraloids, Inc, Wilton NH) by preparing the steroid in redistilled ethanol (1 mg/mL) and then making serial dilutions so as to create a 5-point standard curve ranging from 0.05 to 100 ng/mL. Standards and 10-20 µl of serum were incubated in individual wells of a 96 well plastic plate for 2 h; color developed after washing for 30 min and OD was measured at 650 nm wavelength. Data was processed using the Softmax analysis program (Molecular Devices, Sunnyvale, CA) in a single assay to provide the data reported. The standard curves were reproducible in this assay, and the intra-assay CV was 11.8%.

Statistical Analyses

Group differences for ACTH and cortisol concentrations across time (Experiments 1, 2 and 4), as well as left and right adrenal weights (Experiment 6), were determined by mixed design repeated measures RM-ANOVA (repeated measures design

with between-groups comparisons). Percent suppression of cortisol due to dexamethasone negative feedback (Experiment 3) and concentration of cortisol in hair (Experiment 5) were determined by one-way ANOVA with Fisher's LSD post-hoc analyses. Chi-squared analysis was used to determine differences in distribution of flattened diurnal cortisol patterns between different stress-sensitivity groups in Experiment 1. A 24-hour cortisol pattern was considered flattened if the peak:nadir ratio was less than 2, a clinical criteria often used as a gross measure of abnormal diurnal cortisol activity (Raff et al., 1998). Differences between groups were considered significant if $p \leq 0.05$. Pearson's r correlation analysis was used to evaluate associations between integrated 24-hour area under the curve (AUC) cortisol levels, concentration of cortisol in hair, adrenal weights, and thymus weights. All statistical analyses were performed with SPSS version 15.0 statistical software (SPSS Inc, Chicago, IL).

2.3 RESULTS

Seven animals were categorized as highly stress-resilient (HSR), five as medium stress-resilient (MSR), and five animals as stress-sensitive (SS).

Experiment 1. Characterization of Baseline Diurnal ACTH and Cortisol Secretion

While most animals showed a normal diurnal pattern of plasma cortisol release over the 24-hour sampling period, others failed to show the typical nighttime suppression of HPA axis activity. Normal (peak:nadir ratio > 2) and flattened (peak:nadir ratio < 2) plasma cortisol release patterns occurred in all three stress-sensitivity (HSR, MSR, and

SS) groups (Figure 2.2), and there was no difference in the distribution of flattened cycles between stress-sensitivity groups (χ^2=1.71, p=0.42). Mauchly's W chi-squared test indicated the data for both plasma ACTH and cortisol analysis violated assumptions for sphericity, and thus the most stringent lower-bound estimate of epsilon was used to correct reported p-values. Lower-bound corrected F-statistics for repeated measures analyses are reported in all experiments unless otherwise stated. RM-ANOVA did not detect a significant interaction of the within-group variable of time of day and the between-group variable of stress-sensitivity group for either plasma concentrations of ACTH ($F_{2,12}$=1.08, p=0.37) or cortisol ($F_{2,10}$=0.76, p=0.50). There was a significant main effect of time of day for plasma cortisol concentrations ($F_{1,12}$=6.78, p=0.03), but only a trend for ACTH ($F_{1,12}$=4.46, p=0.06). However, there was not a main effect of stress-sensitivity group on plasma concentrations of ACTH ($F_{2,12}$=0.05, p=0.95) or cortisol ($F_{2,10}$=1.09, p=0.37) over the 24-hour period (Figure 2.3). Integrated 24-hour area under the curve (AUC) was also calculated for both ACTH and cortisol, using the equation $AUC=\Sigma((Cp_1+Cp_2)/2)*(t_2-t_1)$, where Cp= plasma concentration of ACTH or cortisol and t= timepoint. There was no difference in the mean ± SEM 24-hour AUC integrated plasma ACTH (SS: 409.31±49.79 pg/mL/24h; MSR: 370.89±44.27 pg/mL/24h; HSR: 362.65±41.71 pg/mL/24h; p=0.75) or cortisol (SS: 478.41±35.91 μg/dL/24h; MSR: 697.42±79.25 μg/dL/24h; HSR: 628.37±78.14 μg/dL/24h; p=0.15) levels between stress-sensitivity groups. There were also no group differences in the mean daytime (1000 h – 1800 h) or nighttime (2200 h – 0600 h) plasma ACTH or cortisol levels (data not shown).

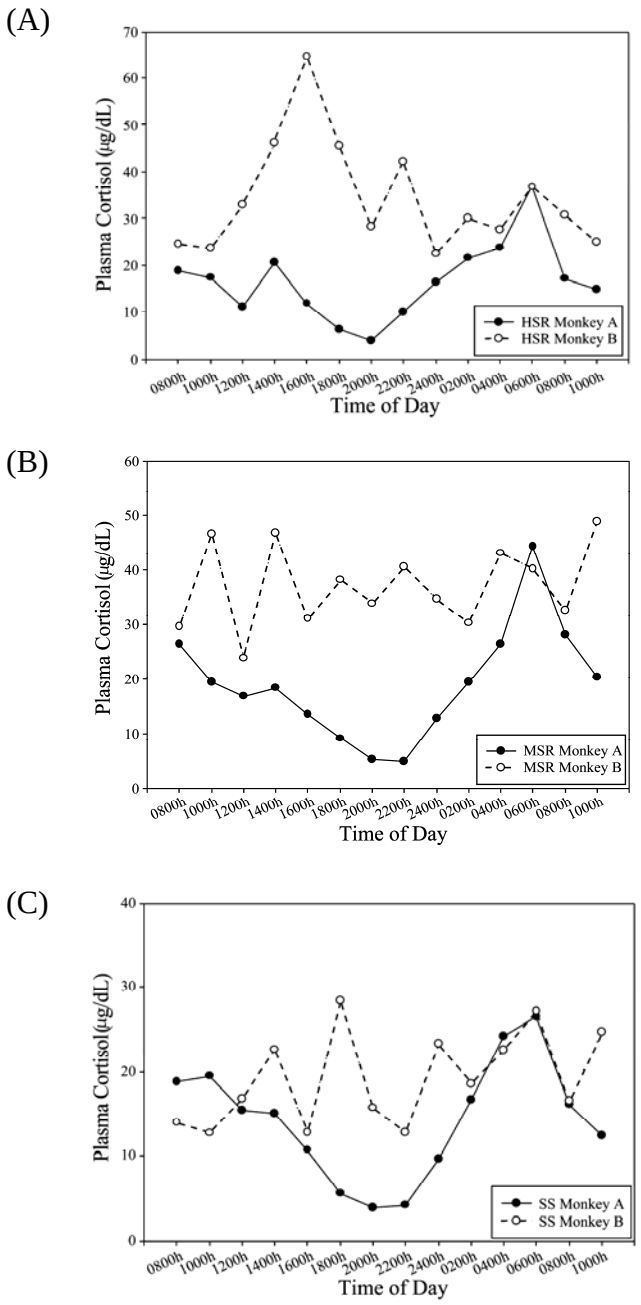
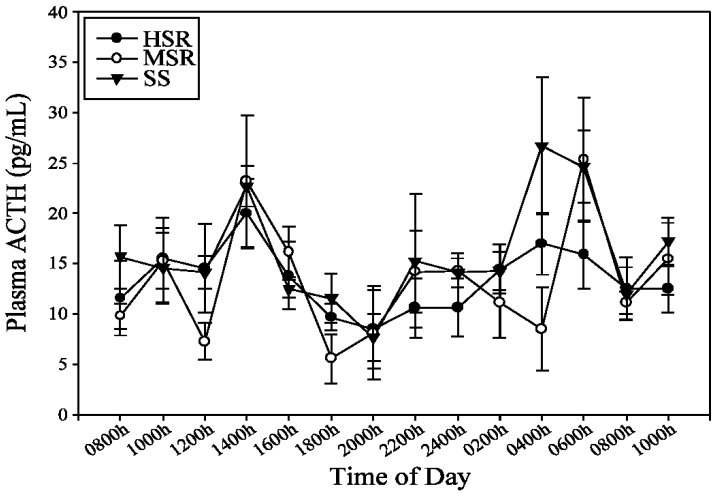


Figure 2.2 Two representative monkeys from each of (A) HSR, (B) MSR, and (C) SS groups, exhibiting either normal diurnal patterns of cortisol secretion (closed circles, solid line) or patterns of cortisol secretion lacking diurnal variation (open circles, dashed line).

(A)



(B)

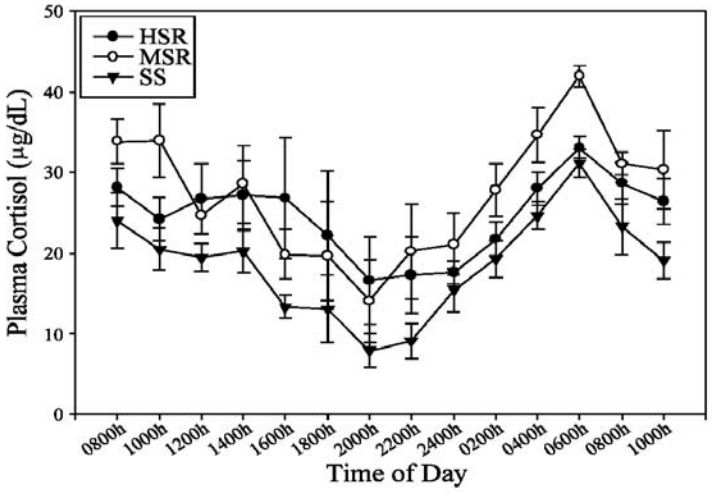
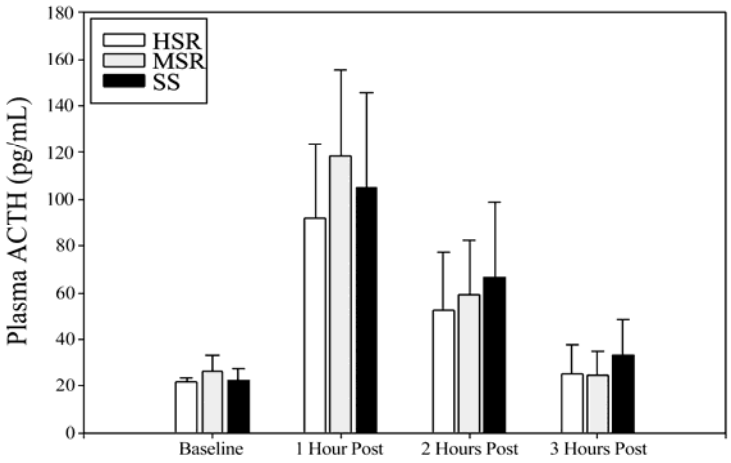


Figure 2.3 Neither plasma ACTH (A) or plasma cortisol (B) release over a normal day differ among SS (closed triangles), MSR (open circles) or HSR (closed circles) monkeys. Values are mean ± SEM.

(A)



(B)

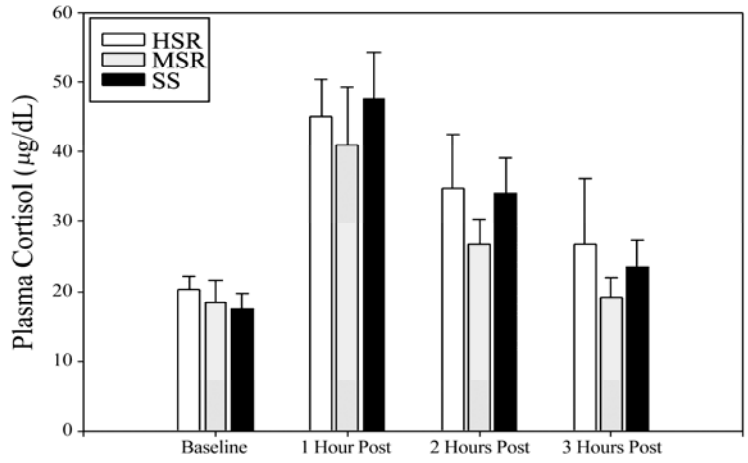


Figure 2.4 Neither plasma ACTH (A) or plasma cortisol (B) release at baseline or at 1, 2, or 3 hours following exposure to an acute psychological stress differ between SS (black bars), MSR (gray bars) or HSR (open bars) monkeys. Values are mean ± SEM.

Experiment 2. HPA Axis Activation to Acute Stress

There was not a significant interaction of time by stress-sensitivity group for either ACTH ($F_{2,13}$=0.29, p=0.75) or cortisol ($F_{2,13}$=.27, p=0.77) release in response to acute psychological stress. There was a main effect of time in both ACTH ($F_{1,13}$=15.68, p<0.01) and cortisol ($F_{1,13}$=17.26, p<0.01) release. The acute stress of leather glove presentation significantly increased ACTH (baseline: 22.46±2.44 pg/mL; 1h Post: 108.23±20.29 pg/mL; t=-4.04, p<0.01) and cortisol (baseline: 18.81±1.38 μg/dL; 1h Post: 44.59±3.83 μg/dL; t=-7.10, p<0.01) by paired t-test comparison for all animals, indicating that the protocol for presentation of the catch glove was an acute stressor (Figure 2.4). RM-ANOVA failed to detect a main effect of stress-sensitivity grouping on plasma ACTH ($F_{2,13}$=0.21, p=0.82) or cortisol ($F_{2,13}$=0.38, p=0.69) response to acute psychological stress (Figure 2.4). There was also no difference in the mean ± SEM 3-hour AUC integrated plasma ACTH (SS: 199±79.17 pg/mL/3h; MSR: 203.05±63.29 pg/mL/3h; HSR: 168.51±62.21 pg/mL/3h; p=0.92) or cortisol (SS: 102.23±10.55 μg/dL/3h; MSR: 86.42±13.31 μg/dL/3h; HSR: 103.44±16.96 μg/dL/3h; p=0.69) levels between stress-sensitivity groups.

Experiment 3. Dexamethasone Suppression of the HPA Axis

Percent suppression of cortisol was calculated by comparing 0800 h baseline levels to plasma samples collected at 0800 h the following day, 15 hours after dexamethasone treatment. There was no difference in the percent suppression of cortisol with dexamethasone treatment between HSR (69.6%±6.4%), MSR (72.2%±14.5%), and SS (55.4%±15.9%; p=0.60) monkeys (Figure 2.5). Clearance rates of dexamethasone

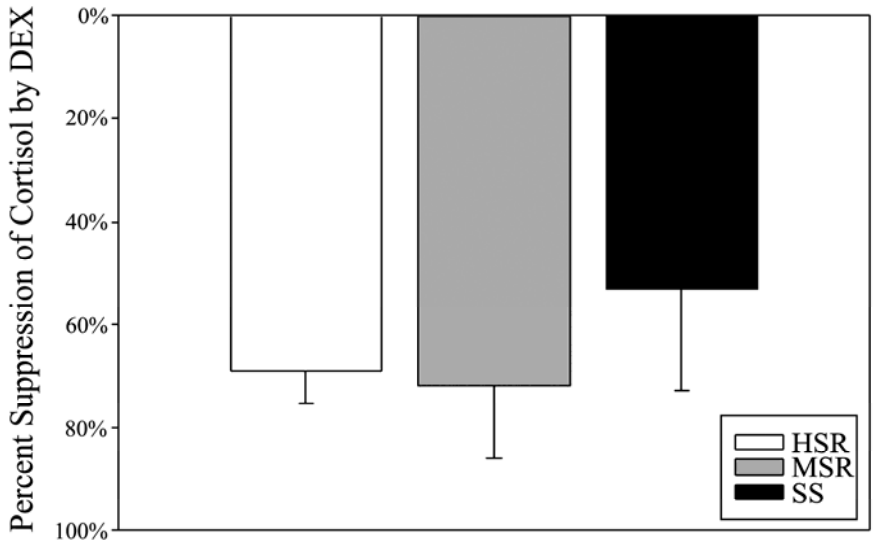


Figure 2.5 The percent suppression of cortisol by exogenously administered dexamethasone (150 μg/kg, i.v.) did not differ in SS (black bars), MSR (gray bars) and HSR (open bars) monkeys. Values are mean ± SEM.

were not different between the groups (HSR: 11.19±0.81 mL/min; MSR: 10.88±0.76 mL/min; SS: 11.09±0.92 mL/min; p=0.97), and there was no correlation between individual clearance rates of dexamethasone and concentration of plasma cortisol collected 15 hours post-dexamethasone (r=0.24, p=0.39, data not shown).

Experiment 4. Characterization of Diurnal ACTH and Cortisol Secretion Following Exposure to Mild Combined Stress

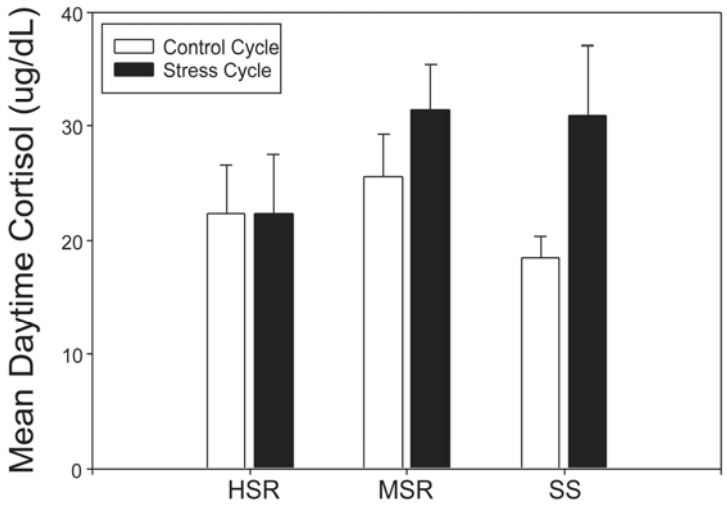
Two HSR monkeys and one SS monkey failed to complete Experiment 4 and as a result, the sample sizes for this experiment were as follows: HSR (n=5), MSR (n=5), and SS (n=4). Twenty-four hour integrated AUC of plasma ACTH and cortisol concentrations did not differ between stress-sensitivity groups, nor within each group when comparing a control menstrual cycle (Experiment 1) to a cycle in which animals were exposed to mild combined stress (Experiment 4; data not shown). This, however, was driven largely by similar ACTH and cortisol concentrations during the nighttime (2200 h to 0600 h) hours. There were no interaction or main effects of condition (control cycle vs. stressed cycle) or stress-sensitivity grouping for ACTH concentration over the 24-hour experimental period (data not shown). RM-ANOVA failed to detect a significant interaction effect of condition by stress-sensitivity group in either mean daytime ($F_{2,11}$=1.59, p=0.25) or nighttime ($F_{2,10}$=0.82, p=0.47) cortisol concentration, nor was there a main effect of either condition ($F_{1,10}$=0.00, p=1.00) or stress-sensitivity group ($F_{2,10}$=0.48, p=0.63) in mean nighttime cortisol levels. However, there was a main effect of condition ($F_{1,11}$=5.22, p=0.04), but not stress-sensitivity group ($F_{2,11}$=0.714, p=0.51) on mean daytime cortisol levels. Within each stress-sensitivity group individually as

detected by a post-hoc paired t-test comparison, HSR monkeys did not differ between a control, non-stressed cycle and a cycle in which they were exposed to mild combined stress (Control: 22.06±5.15 μg/dL; Stress: 22.44±5.08 μg/dL; t=-0.06, p=0.95). In contrast, both MSR monkeys (Control: 25.60±3.56 μg/dL; Stress: 31.44±4.01 μg/dL; t=-1.89, p=0.12) and SS monkeys (Control: 18.35±1.87 μg/dL; Stress: 30.95±6.13 μg/dL; t=-2.83, p=0.06) showed a trend towards increased circulating cortisol, when the control and stressed cycles (Experiment 1 vs. Experiment 4) were compared. This increase was specific to the daytime hours (1000 h-1800 h; Figure 2.6A). Because both MSR and SS groups appeared to have increased cortisol activation in response to the move+diet, and because both groups showed a physiological effect of stress on the reproductive axis (i.e., both groups showed at least one anovulatory menstrual cycle when stressed), the groups were combined (n=9) to make a single comparison with the HSR group (n=5; Figure 2.6B). When analyzing animals that became anovulatory in response to stress (MSR+SS) vs. animals that did not have any reproductive problems (HSR), the MSR+SS group had significantly higher mean daytime cortisol on the day they were moved to a new room and put on a mild diet, as compared to a control menstrual cycle (Control: 22.38±2.39 μg/dL; Stress: 31.22±3.27 μg/dL; t=-3.26, p=0.01), while the HSR group did not.

Experiment 5&6. Measurement of Hair Cortisol and Adrenal Weights

The concentration of cortisol found in hair collected at the end of all experimental protocols did not differ between HSR (93.19±9.71 pg/mg), MSR (113.36±10.61 pg/mg), or SS (100.33±20.71 pg/mg) animals by one-way ANOVA (F=0.68, p=0.52; Figure 2.7). Adrenal tissue weight data met assumptions for sphericity, and RM-ANOVA failed to

(A)



(B)

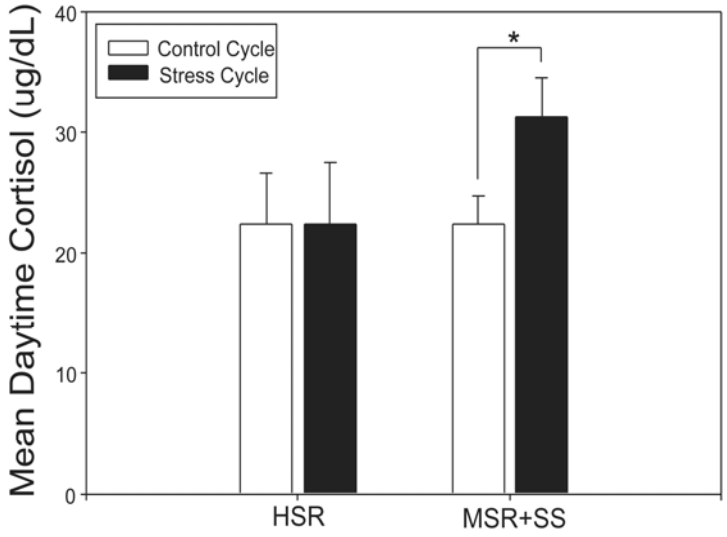


Figure 2.6 Mean levels of plasma cortisol measured during daytime hours (1000h to 1800h) in the early follicular phase of both a control, non-stressed menstrual cycle (open bars) and in the same phase of a cycle with exposure to mild combined stress (move+diet) in (A) HSR, MSR, and SS monkeys, and in (B) in HSR versus animals that became anovulatory in response to mild combined stress (MSR+SS). Values are mean ± SEM.

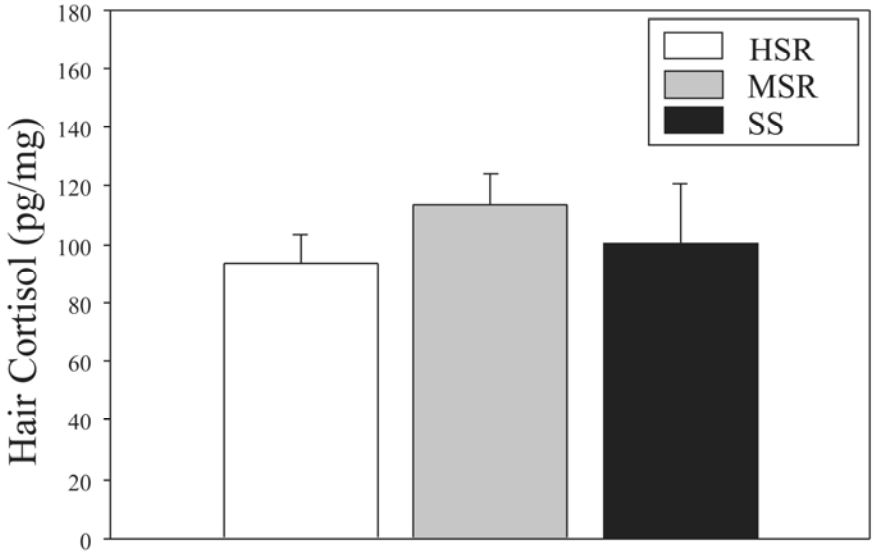


Figure 2.7 Concentration of cortisol hair collected from HSR (open bar), MSR (gray bar) and SS (black bar) monkeys. Values are mean ± SEM.

detect a significant interaction of adrenal side (left vs. right) by stress-sensitivity group ($F_{2,14}$=0.49, p=0.62). There was a main effect of side, in that left adrenal glands weighed more than right ($F_{1,14}$=79.48, p=0.00), due to differences between the left and right adrenal glands in anatomical location and blood supply (Netter, 1981). However, there was not a main effect of group, as post-mortem adrenal weights did not differ between stress-sensitivity groups with respect to left (HSR: 0.38±0.02g; MSR: 0.36±0.04g; SS: 0.41± 0.01g; p=0.50) or right (HSR: 0.29±0.02g; MSR: 0.30±0.02g; SS: 0.34±0.02g; p=0.29) adrenal weights (Figure 2.8).

Pearson's r correlational analysis did not detect a significant correlation of 24-hour integrated AUC levels of cortisol measured in Experiment 1 with left adrenal weight (r=-0.01, p=0.96), right adrenal weight (r=-0.16, p=0.55), nor concentration of cortisol found in hair (r=0.26, p=0.35). Likewise, hair cortisol concentrations were not correlated with either left (r=0.05, p=0.87) or right (r=10, p=0.73) weights. Post-hoc assessment of thymus weights also recorded at sacrifice also did not correlate with 24-hour integrated cortisol (r=0.17,p=0.60), left adrenal weight (r=-0.16, p=0.61) or right adrenal weight (r=0.05, p=0.87). However, there was a significant correlation between thymus weight and concentration of cortisol in hair (r=-0.59, p=0.04).

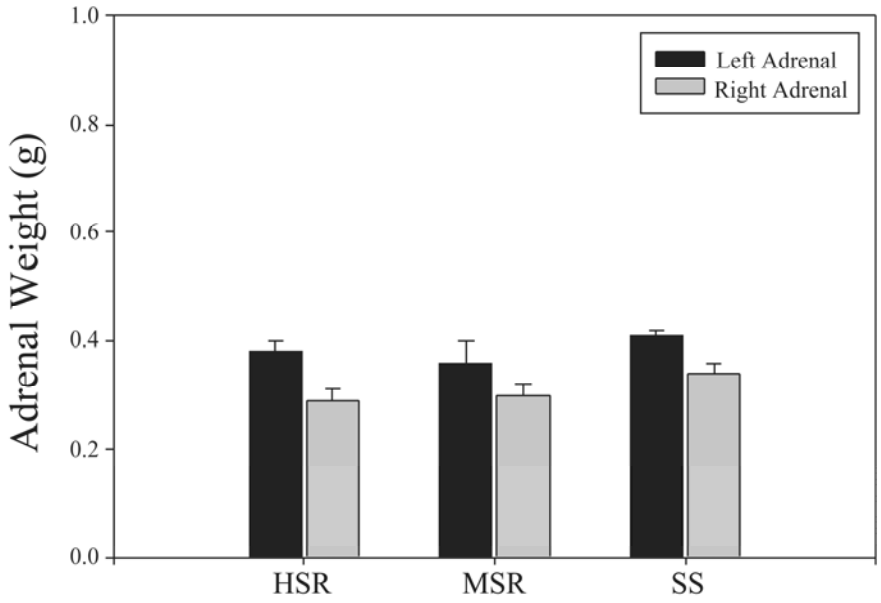


Figure 2.8 Weights of left (black bars) and right (gray bars) adrenal glands from HSR, MSR and SS monkeys. Values are mean ± SEM.

2.4 DISCUSSION

In five different physiological measures designed to characterize activation of the HPA axis in control, non-stressed conditions, I did not find any evidence that activity of the HPA axis is associated with sensitivity to stress-induced reproductive dysfunction. However, monkeys that develop stress-induced menstrual cycle disturbances (i.e. MSR and SS monkeys) do secrete more cortisol, but not ACTH, than HSR monkeys in the specific condition of acute exposure to mild combined psychosocial + metabolic stress. This increase was specific to the daytime when the monkeys could see other novel animals in the room, which might have been a greater psychosocial stressor, as this increase disappeared by evening. These findings indicate that increased activation of the HPA axis only occurs in very limited circumstances in animals that are more sensitive to stress-induced reproductive dysfunction compared to more stress-resilient individuals.

While some studies have reported that women diagnosed with stress-induced amenorrhea have slightly elevated cortisol concentrations in plasma (Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001; Lindahl et al., 2007) and in CSF (Brundu et al., 2006) as compared with eumenorrheic women, not all have reported this finding (Couzinet et al., 1999). Additionally, incongruity with regard to the timecourse of diurnal cortisol elevation (Suh et al., 1988; Berga et al., 1997; Lindahl et al., 2007), and the finding that CRH is not elevated in the CSF of women with this disorder (Berga et al., 2000), complicates the interpretation of these reports. Thus, there is insufficient data to suggest that elevated activity of the HPA axis is causal in the suppression of reproductive function in women with stress-induced amenorrhea. Women with stress-induced amenorrhea report more stress in their lives compared to

eumenorrheic women (Marcus et al., 2001; Bomba et al., 2007). Therefore, it may be that in situations where women with stress-induced amenorrhea are more stressed they have higher cortisol secretion than normally cycling women, although their baseline cortisol secretion is not elevated. Such a mechanism would account for the reported variability in finding that women with stress-induced amenorrhea have elevated cortisol secretion. Interestingly, in this study I found this same pattern of cortisol secretion in stress-sensitive female monkeys. Stress-sensitive monkeys do not have elevated baseline cortisol secretion, but they are more stressed by mild psychosocial plus metabolic stress and show elevated cortisol in this specific situation.

The finding that monkeys with increased sensitivity to stress-induced reproductive dysfunction show increases in mean daytime cortisol secretion following exposure to this paradigm of mild combined stress supports the hypothesis that *responsivity* of the HPA axis to these types of synergistic stressors could be involved in the etiology of stress-induced reproductive dysfunction. If this is indeed true, such an increased responsivity of the HPA axis would be in reaction to a very specific combination of mild combined psychosocial + metabolic stress, as HPA axis response to an acute psychosocial stressor (Experiment 2) did not differ between stress-sensitivity groups. However, the studies designed to measure baseline activation of the HPA axis, including the measures of more chronic HPA axis activity (i.e. diurnal ACTH and cortisol secretion in a non-stressed menstrual cycle, concentration of cortisol in hair, and adrenal weight), indicate that baseline activation of the HPA axis is *not* correlated with sensitivity to stress-induced reproductive dysfunction in female macaques. Because there was only one circumstance in which HPA activity differed between stress-sensitivity

groups, the issue of whether there was adequate power to detect a difference between stress-sensitivity groups if one existed must be considered, despite the preliminary power estimates of 84-93% for this study. To further examine this issue of whether there was adequate power to detect group differences in HPA axis activity between stress sensitivity groups, I attempted multiple methods of data analysis, grouping animals according to those that develop menstrual cycle disturbances (MSR+SS) versus those that do not (HSR) as I did in Experiment 4 to increase the sample size of comparison groups. However, such analyses still yielded no group differences in the measures of baseline activation of the HPA axis. Recalculation of power analysis using the measurement variabilities observed in this study and a power estimate of .80 determined that I would need a sample size of 658 animals to detect differences between groups in diurnal HPA axis activity, 86 animals to detect differences in acute HPA axis activation, 34 animals to detect a difference in dexamethasone suppression, 230 animals to detect differences in cortisol concentration in hair cortisol, and 108 animals to detect differences in adrenal weight. These calculations showing that large numbers of animals would be needed to detect significant group differences are in corroboration with the majority of the experiments in this study finding no association between sensitivity to stress-induced reproductive dysfunction and HPA axis activation, despite a selective activation of cortisol in stress-sensitive monkeys in response to mild combined stress. These findings support the conclusion that differences in HPA axis activation are not the primary mechanism underlying stress-induced reproductive dysfunction in stress-sensitive monkeys. This is reinforced by the fact that our laboratory has previously reported

differences in the baseline activation of the serotonergic system between stress-sensitivity groups with a sample size of 3-4 monkeys/group (Bethea et al., 2005a,b).

This study utilized a relatively new measure of integrated, chronic HPA axis activity by measuring the concentration of cortisol found in hair (Davenport et al., 2006). Hair cortisol concentrations have been shown to significantly correlate with integrated 24-hour AUC urinary cortisol levels (Sauvé et al., 2007) and self-reported measures of perceived stress (Kalra et al., 2007). In this study, however, I did not find a correlation between concentration of cortisol in hair and any measure of HPA axis activity. It is possible that concentrations of ACTH and cortisol in biological fluids such as plasma, saliva or urine, which exhibit diurnal and environmentally-induced fluctuations, may show greater variance than cortisol measures in hair, and because the variances of hair cortisol levels and other physiological measurements would need to be related in order to show a correlation, we might not expect such a relationship in this study. Interestingly, I did find a significant inverse correlation between hair cortisol levels and post-mortem weight of the thymus gland, consistent with previous data indicating that exposure to chronic stress or high levels of exogenous glucocorticoids can cause thymic atrophy (Myers et al., 2005). This *a posteriori* finding provides evidence that hair cortisol is a good integrated measure of chronic stress load, while moment to moment variation in ACTH and cortisol secretion may not provide ideal integrated measures of HPA axis activity.

The majority of the evidence presented herein found that activity of the HPA axis was not correlated with sensitivity to stress-induced reproductive dysfunction. This finding did not support my hypothesis that SS monkeys would have elevated chronic

basal activation of the HPA axis. The hypothesis that HPA axis activity plays a causal role in sensitivity of the reproductive axis to stress was based in part on the results of previous studies from our laboratories that showed that both MSR and SS monkeys have increased CRH gene expression in the caudal PVN, amygdala and thalamus as compared to HSR monkeys (Centeno et al., 2007b). Both glucocorticoids and CRH, itself, have been shown to be capable of suppressing reproductive axis activity in some circumstances. Glucocorticoids have been shown to suppress activity of the reproductive axis only if the elevated circulating levels are *high in concentration* and *chronic* in nature, a finding replicated in rodents (Smith et al., 1971; Baldwin & Sawyer, 1974; Baldwin, 1979; Ringstrom & Schwartz, 1985; Kamel & Kubajak, 1987), monkeys (Plant et al., 1983; Dubey & Plant, 1985; Sapolsky, 1985), and in humans (Boccuzzi et al., 1975; Luton et al., 1977). The levels of elevated cortisol concentrations that have been reported in women with stress-induced amenorrhea (Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001; Lindahl et al., 2007) are only moderately elevated and are not in the high range known to be directly suppressive to activity of the reproductive axis. Interestingly, in the one circumstance in which I found differences in cortisol activation to mild psychosocial + metabolic stress in SS+MSR versus HSR monkeys, this was also a very small physiological difference. On the other hand, elevated CRH has been shown to more acutely suppress activity of the reproductive axis (Rivier et al., 1986; Xiao & Ferin, 1988; Xiao et al., 1989; Helmreich et al., 1993). Interestingly, the adrenal glands and endogenous cortisol are not required for acute CRH-induced suppression of the HPG axis (Xiao et al., 1989). Furthermore, it has also been reported that short-term ACTH infusions do not interfere with normal pulsatile LH

secretion in the macaque (Xiao & Ferin, 1988). Therefore, if CRH is involved in suppression of the reproductive axis, and/or sensitivity to stress-induced reproductive dysfunction, this signaling system could occur via HPA axis-independent mechanisms, as CRH has numerous neurotransmitter functions outside its role as neuroendocrine regulator of the HPA axis.

CRH neuronal projections from both the hypothalamus and amygdala serve to regulate not only activation of the HPA axis, but other autonomic and behavioral functions and both brain regions have populations of CRH neurons possessing bidirectional connectivity with most of the limbic and autonomic brain areas (Swanson, 1987). I report here, using five different physiological measures, that HPA axis activity is not elevated in individuals sensitive to stress-induced reproductive dysfunction. Therefore, it is not likely that the increased CRH gene expression reported in the caudal PVN and amygdala of stress-sensitive individuals (Centeno et al., 2007b) is associated with increased activity of CRH neuroendocrine neurons projecting to the median eminence and stimulating ACTH and cortisol release. Alternatively, the increased CRH gene expression in the caudal PVN may be in neurons that act in a non-neuroendocrine manner to regulate other neurotransmitter systems that also mediate function of the reproductive axis, including norepinephrine, dopamine, serotonin, γ-aminobutyric acid (GABA) and glutamate (Tilbrook et al., 2002; Dobson et al., 2003).

The serotonergic system is at least one neurotransmitter network that appears to show marked physiological differences between female monkeys sensitive and resilient to stress-induced reproductive dysfunction. Work from the Cameron and Bethea laboratories have shown that stress-sensitive individuals have suppressed physiological

release of serotonin (Bethea et al., 2005a), as well as fewer serotonergic cells and low expression of a number of genes in the serotonin pathway, including FEV1, TPH2, SERT, MAO-A and MAO-B, in the dorsal raphe nucleus (Bethea et al., 2005b). Moreover, treatment with a selective serotonin reuptake inhibitor, citalopram, increases ovarian steroid hormone secretion in SS monkeys (Cameron et al., 2004). The CRH system is a regulator of serotonergic neurons in the dorsal raphe nucleus (Cole & Sawchenko, 2002). Thus, it is possible that in stress-sensitive monkeys elevated CRH may be leading to the suppression of serotonin neurons in the raphe nucleus. In fact, studies in the rat indicate that the caudal PVN does not project to the median eminence to stimulate ACTH and cortisol release in the HPA axis, but rather projects to the limbic system and brainstem regions (Sawchenko & Swanson, 1982). Data described in Chapter 5 of this dissertation suggests that stress-sensitive monkeys indeed have greater immunocytochemical staining of CRH fibers in the dorsal raphe nucleus compared with more stress-resilient monkeys (Herod et al., 2008). Thus, increased activity of CRH neurons in the caudal PVN may be linked to sensitivity of the reproductive axis to stress, not through increased activation of the HPA axis, but by inhibiting serotonin neuronal activity in the raphe nucleus.

In this study I did not find a relationship between activity of the HPA axis and sensitivity to stress-induced reproductive dysfunction in five different physiological measures of HPA axis activity. These findings present evidence in opposition to my hypothesis that activation of the HPA axis is a primary neural mechanism underlying sensitivity to stress-induced reproductive dysfunction. However, the singular finding that monkeys with increased sensitivity to stress-induced reproductive dysfunction show an

increase in cortisol secretion following exposure to mild combined stress dissents from the rest of the data discussed in this chapter. Although it is unlikely given the overwhelming lack of an association between HPA axis activity and stress-sensitivity discussed in these studies, the findings of Experiment 4 suggest that responsivity of the HPA axis to these specific types of synergistic stressors (mild psychosocial + metabolic stress) might be involved in the etiology of stress-induced reproductive dysfunction. Therefore, in studies reported in Chapter 3 I utilized pharmacological studies to test whether responsivity of the HPA axis to synergistic mild combined stressors is causal in the suppression of reproductive dysfunction in more stress-sensitive monkeys.

Chapter 3

TREATMENT WITH A CRH-R1 ANTAGONIST PREVENTS STRESS-INDUCED SUPPRESSION OF THE CENTRAL NEURAL DRIVE TO THE REPRODUCTIVE AXIS

3.1 INTRODUCTION

Stress-induced amenorrhea is a form of stress-induced infertility in which the underlying cause is thought to be reduced central hypothalamic gonadotropin releasing hormone (GnRH) drive to the reproductive axis (Liu et al., 1990). This disorder has historically been a diagnosis of exclusion after ruling out other organic causes of amenorrhea. However, the widespread observation that women with stress-induced amenorrhea have decreased luteinizing hormone (LH) pulse frequency (Crowley et al., 1985; Reame et al., 1985; Khoury et al., 1986; Suh et al., 1988; Berga et al., 1989; Giles and Berga, 1993; Berga et al; 1997; Laughlin, Dominguez, & Yen, 1998; Couzinet et al., 1999; Marcus et al., 2001; Bomba et al., 2007), and therefore presumably slowed pulsatile GnRH, has recently allowed a diagnosis of inclusion with this common symptom (Giles and Berga, 1993; Marcus et al., 2001).

Stress-induced amenorrhea has been attributed to a combined effect of mild psychosocial stress + metabolic stress, acting to suppress GnRH drive to the reproductive axis and normal reproductive function (Giles & Berga, 1993; Berga et al., 1997; Marcus et al., 2001). Our laboratory has developed a non-human primate model of sensitivity to stress-induced reproductive dysfunction, in which female cynomolgus macaques (*Macaca fascicularis*), that have 28-day menstrual cycles like human women, are

exposed to a combination of mild psychosocial stress plus a moderate metabolic stress of reduced calorie intake, either with or without exercise (Cameron, 1997; Cameron et al., 1998; Cameron, 2000; Bethea et al., 2005a,b; Centeno et al., 2007a,b,c; Williams et al., 2007; Bethea et al., 2008; Herod et al., 2008). This model was designed based on clinical descriptions of the stressor (psychogenic and metabolic) levels in women seeking treatment for stress-induced amenorrhea (Marcus et al., 2001). Using this monkey model, previous work in our laboratory has shown that monkeys differ in their sensitivity to stress-induced reproductive dysfunction (Cameron, 1997; Cameron, 2000; Williams et al., 2007; Bethea et al., 2008), with some monkeys showing no impairment of reproductive function following exposure to mild combined stress (i.e., are "stress-resilient"), and others showing marked sensitivity of the reproductive axis to stress (i.e. are "stress-sensitive"). The fact that the same external mild stressors cause differential effects on the reproductive axis in individual monkeys suggests that stress-sensitive monkeys might have an increased allostatic load of mild combined stress on reproductive function, as compared to more stress-resilient monkeys. These differences in stress-sensitivity are stable characteristics of an individual, and can be predicted by a number of physiological (Cameron et al., 1998) and behavioral markers (Herod et al., 2006).

The exact mechanism by which combined psychosocial and metabolic stresses suppress GnRH neuronal drive to the reproductive axis in women with stress-induced amenorrhea is still unknown, although elevated activity of the hypothalamic-pituitary-adrenal (HPA) axis, as regulated by central corticotropin-releasing hormone (CRH) neurons, has been implicated in several studies of stress-induced amenorrhea (Biller et al., 1990; Berga et al., 1997; Meczakalski et al., 2000; Kondoh et al., 2001; Brundu et al.,

2006; Lindahl et al., 2007). In Chapter 2 of this dissertation, I reported that activity of the HPA axis does not differ between monkeys with different sensitivities to stress-induced reproductive dysfunction. However, animals that show reproductive suppression following stress (medium stress-resilient; "MSR" + stress-sensitive; "SS" monkeys) do have an increase in cortisol secretion following acute exposure to a mild psychosocial + metabolic stress paradigm during the daytime hours only, when monkeys can see other novel monkeys and are perhaps more affected by this psychosocial stressor.

The physiological response of the HPA axis to stress is thought to consist of three distinct phases: initiation, maintenance and recovery (Coste et al., 2001; Figure 3.1). An individual's progression through these phases of this stress response is in large part mediated by the two known receptors for CRH, the neurohormone released from the hypothalamus into the median eminence to initiate downstream activation of the HPA axis. CRH-R1 and CRH-R2 are both G-protein coupled transmembrane receptors with non-overlapping distribution throughout the brain and central nervous system (Vale et al., 1997). Based on studies in mice lacking one or the other of these two known CRH receptors, CRH-R1 is thought to be involved in the initiation phase of the stress response, while CRH-R2 may play an important role in maintaining and terminating this response during the recovery phase (Coste et al., 2001). CRH-R1 knockout mice have extremely low levels of plasma corticosterone that do not exhibit normal diurnal fluctuations, as well as pronounced atrophy of the adrenal glands (Smith et al., 1998; Timpl et al., 1998), while CRH-R2 knockout mice show prolonged elevation of adrenocorticotropic hormone (ACTH) and corticosterone following stress exposure (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000). Additionally, CRH-R1 knockouts do not activate the HPA

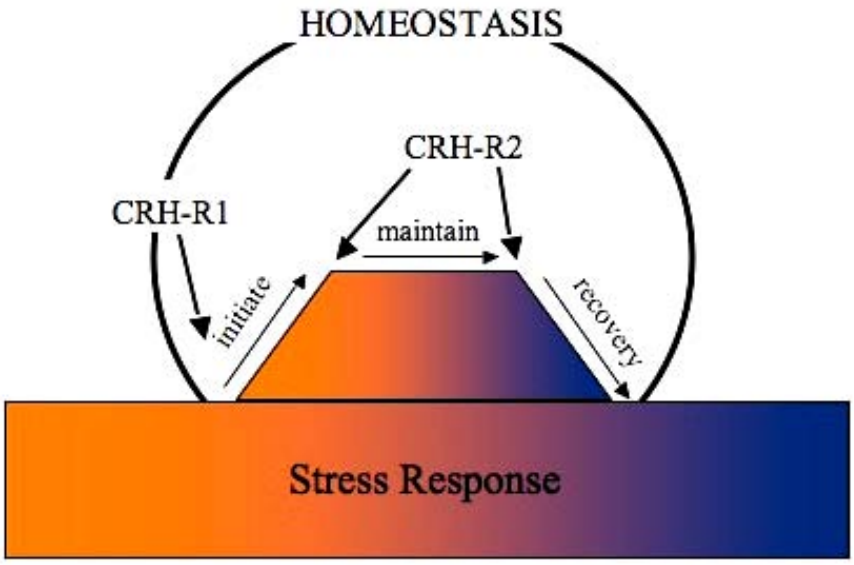


Figure 3.1 Schematic of the physiological stress response indicating three distinct phases: initiation, maintenance, and recovery, and the proposed roles for CRH-R1 and CRH-R2 in mediating these phases. Adapted from Coste et al., 2001.

axis following stress exposure, as ACTH and corticosterone levels measured after restraint or forced-swim stress are comparable to baseline (Smith et al., 1998; Timpl et al., 1998). As it appears that the CRH-R1 receptor is integral in mounting a physiological response to stress, blocking the CRH-R1 receptor with a specific antagonist would be expected to attenuate the normal activation of the adrenal axis in response to acute stress. It would thus be of interest to test the ability of a selective CRH-R1 antagonist to improve reproductive function in female monkeys that show sensitivity to stress-induced reproductive dysfunction, as these monkeys have increased cortisol secretion following exposure to mild combined stress.

Antalarmin, originally described by Chen (1994), is a small lipophilic non-peptide compound that has been shown to penetrate the blood-brain barrier (Hsin et al., 2002; Habib et al., 2000) and selectively block CRH-R1 receptors. Antalarmin is similar to another CRH-R1 antagonist, CP 154,526 (Chen et al., 1994) but exhibits increased lipophilicity and affinity for the CRH-R1 receptor (Hsin et al., 2002). Despite the possibility of promising clinical usage, the reported effects of antalarmin on HPA axis activation and stress-induced behaviors in rodent models of anxiety are inconsistent. Some studies have reported that antalarmin blocks stress-induced ACTH and cortisol release (Bornstein et al., 1998; Webster et al., 1996), while others have found no effect (Wong et al., 1999). Interestingly, several groups have reported that antalarmin blocks stress-induced behaviors, without suppression of the HPA axis (Myers et al., 2005; Deak et al., 1999). It has become apparent that both the species and strain of the animal model and behavioral measurement used are critical, as the effect of antalarmin on restraint stress-induced corticosterone release has been reported to be dependent upon rodent

strain (Grakalic et al., 2006), and the use of various testing procedures yield different outcomes as to the effects of antalarmin on stress-related behaviors (Nielsen et al., 2004).

Research on the effects of antalarmin on HPA axis activation and stress-related behavior has been more limited in primate species. Habib and colleagues (2000) reported that orally administered antalarmin (20 mg/kg) attenuated social stress-induced increases in ACTH, cortisol, and stress-related behaviors (fear grimacing, open mouth threats, teeth grinding, freeze behavior, urination and defecation) in male rhesus monkeys. This finding was recently corroborated in the marmoset (French et al., 2007). However, Ayala and colleagues (2004) found that orally-administered antalarmin did not attenuate stress-induced elevations in ACTH or cortisol, although it did reduce stress-related behaviors. Intravenously-administered antalarmin at various doses has only been studied by one group (Broadbear et al., 2002; Broadbear et al., 2004). In these studies performed with catheterized male rhesus macaques, intravenously administered antalarmin was reported to reduce CRH-induced elevations in ACTH, but not cortisol, at doses of 1.0 and 3.2mg/kg. Higher i.v. doses of antalarmin did not attenuate elevations in ACTH or cortisol, but rather increased the cortisol response to a CRH challenge (Broadbear et al., 2004). The first study described in this chapter (Experiment 1) was a pilot study designed to test the effect of intravenously administered antalarmin on the HPA axis response to psychological stress and directly compare it to the effects of orally-administered antalarmin in female cynomolgus monkeys.

In addition to a pilot dose response study to determine effective dosing and route of administration of antalarmin, I designed a second study to determine whether there was an acute effect of stress on reproductive function, as previous studies of monkeys

exposed to psychosocial + metabolic stress had only looked at long-term effects of stress on reproductive function (i.e., the ability of stress to impair menstrual cyclicity), and it was not feasible to administer antalarmin over a period of an entire menstrual cycle. Previous studies in other animal models of stress-induced reproductive dysfunction had measured changes in LH pulse frequency in response to various stressors (Rivier et al., 1986; Xiao & Ferin, 1988; Xiao et al., 1989; Helmreich et al., 1993), and decreased LH pulse frequency is a common symptom in women with stress-induced amenorrhea (Crowley et al., 1985; Reame et al., 1985; Khoury et al., 1986; Suh et al., 1988; Berga et al., 1989; Giles and Berga, 1993; Berga et al; 1997; Laughlin, Dominguez, & Yen, 1998; Couzinet et al., 1999; Marcus et al., 2001; Bomba et al., 2007). Therefore, in Experiment 2 I tested the hypothesis that there is a greater suppression of pulsatile LH secretion following acute exposure to a mild combined stress in monkeys that showed elevated cortisol secretion in response to psychosocial + metabolic stress (i.e., MSR+SS monkeys) vs. HSR monkeys. In Experiment 3, I tested the hypothesis that pre-treatment with the selective CRH-R1 antagonist, antalarmin, would prevent the hypothesized suppression of LH pulse frequency in response to stress in MSR+SS monkeys, presumably by suppressing activation of the HPA axis.

3.2 METHODS AND MATERIALS

Animals

Fifteen female cynomolgus monkeys (*Macaca fascicularis*), 7-10 years of age, were utilized for this study, and were from the same group of animals utilized in Chapter

2 of this dissertation. Monkeys were housed at the Oregon National Primate Research Center in individual stainless steel cages in a temperature-controlled room (23 ± 2°C) with lights on for 12 h per day (0700 h to 1900 h). Animals were fed two meals a day consisting of four high-protein monkey chow biscuits (no. 5047, jumbo biscuits; Ralston Purina Co., St. Louis, MO) at 0930 h and 1530 h, and a supplement of one-quarter piece of fresh fruit was provided with each afternoon meal. Animals had their vaginal area swabbed daily to check for menses. The first day of menses was designated Day 1 of a menstrual cycle. Food intake, measured just before the next meal was fed, was recorded for each meal and weight was measured weekly. All protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center.

Catheterization

Each monkey had a chronic indwelling venous catheter placed in a subclavian vein under isoflurane anesthesia (VedCo, St. Joseph, MO). At surgery, the tip of the catheter was positioned in the right atrium of the heart and the free end was routed subcutaneously to the back and threaded through a small cutaneous incision between the scapulae. To protect the catheter line, each animal wore a fitted nylon jacket connected to a 36-inch flexible metal tether that attached to a swivel mounted to the top of the monkey's cage (Cameron & Nosbisch, 1991). Silastic tubing extended from the top of the swivel through a hole in the wall to a sampling syringe and stopcock in the adjacent room. This catheter system allowed for blood sampling and intravenous infusions without sedating or disturbing the animal, while allowing the monkey free range of

motion within the cage. Catheter lines were kept patent with a constant infusion of physiological saline (Baxter Healthcare, Deerfield, IL) containing heparin sodium (4 IU/mL) at a rate of approximately 100 mL/day. Weekly inspections of catheter systems and replacement of a sterile dressing covering the catheter exit site were performed under Ketaset (ketamine hydrochloride, 10 mg/kg i.v., Wyeth, Madison, NJ) to keep the exit site aseptic and prevent infection. Ketamine was never administered within 24 hours preceding an experimental procedure.

Study Design

Prior to the studies presented in Chapters 2 and 3, monkeys had been maintained in a stable social environment on their standard diet and had shown at least one normal menstrual cycle (CONTROL CYCLE I), indicted by a peak luteal phase progesterone of 2 ng/mL or higher (indicating ovulation) and a normal cycle length (25-38 days; Williams et al., 2001a,b). Figure 3.2 depicts the experimental timeline of the studies in this chapter. Monkeys were tested in two cohorts, the first (n=7) being tested in all studies presented in Chapters 2 and 3 between 2005 and 2007, and the second (n=8) being tested between 2007 and 2008 (2 of the original 17 animals utilized in Chapter 2 experiments did not progress to the studies undertaken in this chapter). Experiment 2, Part 1 (LH pulse frequency in a control non-stressed menstrual cycle; as described below) was conducted prior to assessment of stress-sensitivity in order to characterize accurate baseline LH pulse frequency measurements. Following completion of Experiment 2 Part 1, the animals underwent categorization for stress-sensitivity of the reproductive axis, the mild combined stress paradigm lasting for a two-cycle duration (STRESS CYCLE I & II)

as described below. When each monkey had exhibited at least one normal menstrual cycle following exposure to the mild combined stress paradigm (CONTROL CYCLE II), Experiment 1 was conducted during the next cycle. Thereafter, before each experiment, each monkey had to show at least one normal menstrual cycle before progressing on to the next planned experiment. For example, if the monkey had a normal menstrual cycle following Experiment 1 (pilot dose response study), Experiment 2 or 3 (Experiments 2 and 3 were counterbalanced for order effects) was conducted on Day 1 of the following cycle. If the animal failed to exhibit a normal cycle following Experiment 1, Experiment 2/3 was postponed until the animal resumed normal cyclicity.

Assessment of Stress-Sensitivity

For each monkey, sensitivity of the reproductive axis to stress was categorized by assessing changes in menstrual cycle length, ovulation, and reproductive hormone secretion when monkeys were exposed to a mild psychosocial + metabolic stress, as described previously (Cameron, 1997; Cameron et al., 1998; Cameron, 2000; Bethea et al., 2005a; Bethea et al., 2005b; Centeno et al., 2007a; Centeno et al., 2007b; Williams et al., 2007; Bethea et al., 2008; Herod et al., 2008). This study was performed after each monkey had been living in its home cage, surrounded by familiar monkeys for several months. To model mild psychosocial stress, monkeys were moved on the first day of their menstrual cycle from their home cage to a single cage in a novel room where they were

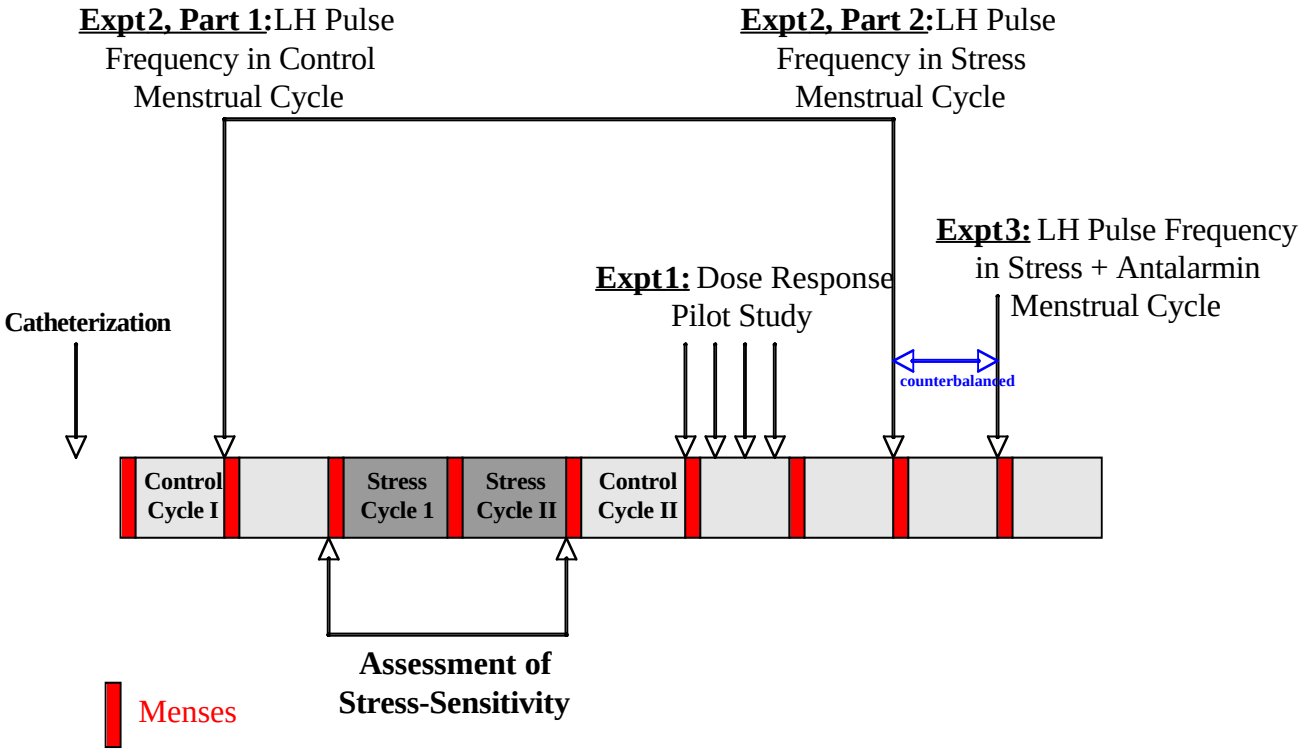


Figure 3.2 Schematic diagram of the experimental timeline of studies detailed in Chapter 3.

surrounded by unfamiliar monkeys. As a metabolic stress, each animal's available caloric intake was reduced by 20%. Blood samples (0.6 mL/sample) were taken every other day to assess reproductive steroid hormone concentrations. Monkeys that mensed within 38 days subsequent to the initiation of stress were moved for a second stress cycle and remained on 20% lower calorie intake.

Animals were categorized as "highly stress-resilient" (HSR) if they presented a normal ovulatory menstrual cycle [25-38 days in length, peak E_2 > 200pg/mL in follicular phase, peak P_4 > 2 ng/mL in luteal phase (Williams et al., 2001a,b)] in *both* Stress Cycle 1 and again in Stress Cycle 2. "Medium stress-resilient" animals (MSR) were defined as those animals that presented a normal ovulatory menstrual cycle in response to Stress Cycle 1, but failed to mense by Day 38 of Stress Cycle 2. Animals that immediately suppressed normal menstrual cyclicity upon exposure to stress (i.e. failed to ovulate and mense within 60 days of Stress Cycle 1) were categorized as "stress-sensitive" (SS). Animals that exhibited disrupted menstrual cycles during this characterization of stress-sensitivity (i.e. MSR and SS monkeys) were allowed to recover normal menstrual cyclicity before resuming further experiments.

Experimental Protocols:

Experiment 1. Comparison of Orally- and Intravenously-Administered Antalarmin on HPA Axis Response to an Acute Psychological Stressor: A Dose Response Pilot Study

Acute Psychological Stress and Blood Collection Protocol

To characterize the intensity and duration of HPA axis activation in response to an acute psychological stressor, leather "catch gloves" were presented to each monkey in a standardized protocol (Helmreich et al., 1993; Rogers et al., 1999). The same psychological stressor was utilized as detailed in Chapter 2, Experiment 2. Following the collection of two baseline samples (0.4 mL/sample, one assayed for ACTH and the other for cortisol), a female person who was not the animal's primary caretaker, wearing a red lab jacket, entered the room and stood next to the monkey's cage. The person presented the leather "catch gloves" to the monkey for a two-minute duration at 15, 30, and 45 min. These gloves are long leather gloves used at the Oregon National Primate Research Center (ONPRC) to hand-catch unanesthetized monkeys. The person wearing the catch gloves manipulated the lock on the cage, touched the tops and sides of the cage, and pretended to initiate capture of the animal, but did not open the cage. All experiments were conducted during the afternoon, between 1300 h and 1600 h, to minimize confounds due to diurnal variations in baseline ACTH and cortisol release. Following the last entry of the person wearing catch gloves, the door to the room was shut for the evening, according to normal nighttime protocol. Blood samples were again taken at 60 min, one hour after the baseline samples were collected.

Blood samples to be assayed for cortisol were collected into heparinized syringes, placed in empty sterile eppendorf tubes on ice and immediately centrifuged at 3000 rpm for 15 minutes at 4°C. Plasma was pipetted into a plastic O-ring storage vial (containing 20 µL of a solution composed of equal volumes of 30% sodium citrate and 1,000 IU/mL sodium heparin, to prevent clotting of plasma proteins) and stored at -20°C until assayed. Blood samples for measurement of plasma ACTH were collected into non-heparinized

syringes, placed in sterile eppendorf tubes containing 20 µL of EDTA (28.8 mg/mL, pH 8.0) on ice and immediately centrifuged at 3000 rpm for 15 minutes at 4°C. Plasma was pipetted into a plastic O-ring storage vial containing 10 µL of aprotinin (500 KIU/mL) and stored at -20°C until assayed. Red blood cells from the sample collected for detection of cortisol (not containing EDTA) were resuspended in sterile heparinized saline and reinfused through the catheter system.

Assessment of Differential Dosing and Administration Routes of Antalarmin

Antalarmin was a generous gift from NIH (Bethesda, MD). Each animal was exposed to the stress protocol of leather catch glove exposure described above in four conditions: no drug (control), with oral antalarmin pre-treatment, and with two doses of i.v. antalarmin pre-treatment. For the oral dose condition, antalarmin was weighed out at 20 mg/kg and the total dose was dissolved into a small piece of fresh fruit, given 90 minutes prior to the baseline blood sample collection. A small piece of fresh fruit, not containing any drug, which was regularly given to the animals at this time of day, was provided during the no drug condition and on i.v. drug condition test days. For i.v. drug conditions, antalarmin was dissolved immediately before use in ethanol/cremophor/sterile water (5:5:90, vol/vol/vol) and filtered using a 0.22 µm filter to ensure sterility. A low i.v. dose of 3.2 mg/kg and a high i.v. dose of 10 mg/kg were tested, based on previous studies utilizing these doses (Broadbear et al., 2002; Broadbear et al., 2004). In both the 3.2 mg/kg i.v. and the 10 mg/kg i.v. dose conditions, the i.v. antalarmin was administered immediately following collection of the baseline blood sample (15 minutes prior to the first entry of the person wearing leather “catch gloves” into the room). The order of the

four testing conditions was randomized for each monkey, in order to avoid a possible confound of acclimation by multiple exposures to the psychological stress.

Experiment 2. Characterization of LH Pulse Frequency in a Control Menstrual Cycle and Following Acute Exposure to Mild Combined Stress

Part 1. Measurement of LH Pulse Frequency in a Control Menstrual Cycle

Blood samples (0.6 mL/sample) were collected into heparinized syringes every 15 minutes via the remote sampling system for eight hours beginning at 0900 h and continuing until 1700 h on Day 5 of a control, non-stressed menstrual cycle. Progesterone was checked in the luteal phase of the preceding cycle to ensure that each animal was having a normal, ovulatory menstrual cycle prior to this experiment. One animal presented normal ovulatory menstrual cycles during the control period (CONTROL CYCLE I) but failed to resume normal menstrual cycles following exposure to the mild combined stress paradigm within a period of six months, and therefore the experiment to characterize LH pulsatility was scheduled after determining that the steroid hormones E_2 and P_4 were low (<50 pg/mL and <0.2 ng/mL, respectively), simulating an early follicular phase. Similar plasma levels of estrogen and progesterone were observed in all monkeys that were sampled on Day 1 of a normal ovulatory menstrual cycle and as such this monkey was included in all analyses. Samples were collected into heparinized syringes and placed in an empty sterile plastic centrifuge tube on ice. Samples were immediately centrifuged at 3000 rpm for 15 minutes at 4°C, and plasma was pipetted into a plastic O-ring storage vial (containing 20 µL of a solution composed of equal volumes of 30% sodium citrate and 1,000 IU/mL sodium heparin to prevent clotting of plasma

proteins) and stored at -20°C until assayed. Red blood cells were sterilely resuspended and reinfused through the remote catheter system. Hematocrit was checked at intervals during the study and remained in the normal physiological range. This experiment was conducted prior to the categorization of stress-sensitivity, in order to accurately characterize LH pulse frequency in a true control menstrual cycle, prior to any exposure to stress.

Part 2. Measurement of LH Pulse Frequency in a Stressed Menstrual Cycle

On Day 1 of another menstrual cycle animals were moved at 0930 h to a cage in a novel room and their available caloric intake was reduced by 20%. Monkeys were studied on Day 1 for this experiment to mimic the effects of mild combined stress on the reproductive axis observed in the assessment of stress-sensitivity paradigm. Plasma concentrations of estrogen and progesterone did not differ between Days 1 and 5 of the early follicular phase. Beginning at 1000 h, blood samples (0.6 mL/sample) were collected for assessment of plasma LH concentration into heparinized syringes every 15 minutes via the remote sampling system continuing until 1800 h that day. Blood samples were collected and processed in an identical manner to that described above. Upon completion of this experiment, the animal's diet was returned to normal and it was kept in the new room to resume normal menstrual cyclicity.

Experiment 3. Characteracterization of HPA Axis Activity and LH Pulse Frequency Following Acute Exposure to Mild Combined Stress with Antalarmin Pre-Treatment

On Day 1 of a normal menstrual cycle after completion of Experiment 1, each monkey was again moved to a single cage in a novel room and placed on a reduced diet (20% reduction in available caloric intake) at 0930 h. The order of Experiment 2, Part 2 and Experiment 3 were randomized, such that some animals were studied in the stress alone condition (Experiment 2, Part 2) first, and others were studied in the stress + antalarmin pre-treatment condition first (Experiment 3), to control for order effects. In Experiment 3 animals were pretreated with 10 mg/kg i.v. antalarmin fifteen minutes prior to the move to the novel room and 20% reduced calorie diet. Intravenous antalarmin solution was dissolved in ethanol/cremophor/sterile water (5:5:90, vol/vol/vol; Habib et al., 2000; Broadbear et al., 2002) and administered through the i.v. catheter at 9:15 a.m. Beginning at 1000 h, blood samples (0.6 mL/sample) were collected into heparinized syringes every 15 minutes via the remote sampling system continuing until 1800 h. Also beginning at 1000 h and continuing every 2 hours until 1800 h, blood samples (0.4 mL/sample) for ACTH and cortisol were collected and processed, to measure the ability of a CRH-R1 antagonist to prevent the increase in cortisol secretion in response to mild combined stress, as was observed in MSR+SS monkeys (see Chapter 2). Samples to be assayed for cortisol were collected and processed in a manner identical to those assayed for LH. Samples to be assayed for ACTH were collected into sterile plastic centrifuge tubes on ice containing 20 μL EDTA to prevent protein degradation. Samples were immediately centrifuged at 3000 rpm for 15 minutes at 4°C, and plasma was pipetted into a plastic O-ring storage vial (containing 10 μL of aprotinin), flash frozen in a dry ice/ethanol bath and stored at -20°C until assayed. At 1400h, a second identical dose of antalarmin was administered, based on literature reporting the half-life of antalarmin to

be 7.82 hours (Habib et al., 2000). The use of this dosage was based on previous literature (Broadbear et al., 2002) and the preliminary results of Experiment 1. Results from the first cohort of animals studied (n=7) indicated that the 10 mg/kg i.v. dose was most effective in suppressing the stress-induced rise in ACTH and cortisol, and animals in this cohort completed physiological experiments and were euthanized before the complete data set for Experiment 1 was collected in the second cohort of monkeys. As such, the higher i.v. dose (10 mg/kg) of antalarmin was used in Experiment 3.

Hormone Assays

Plasma LH was measured by RIA at the University of Pittsburgh assay core as previously described (Williams et al., 2001) using recombinant cynomolgus monkey LH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) as a standard. The sensitivity of the LH assays was 0.063 ng/mL and the intra- and inter-assay coefficients of variation for the LH assays used in these studies, as calculated from three low, medium, and high concentration serum pool controls run in each assay, were 3.84% and 15.36%, respectively. LH pulses were identified using the Pulsar algorithm developed by Merriam and Wachter (1982), using the following G-values: G(1): 4.40, G(2): 2.60, G(3): 1.92, G(4): 1.46, and G(5): 1.13. For all Pulsar analyses, values below the level of assay detectability were assigned the minimal detectable LH concentration in the assay. The rise above baseline in each Pulsar-defined pulse was used in quantifying pulse amplitude. Pulse frequency was calculated as the number of Pulsar-defined pulses in 8 h. Mean LH concentrations were calculated by taking the mean of all LH values collected over the 8-hour sampling period.

Plasma ACTH and cortisol, as well as estrogen and progesterone, were measured with the Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL) in the Endocrine Services Laboratory (ESL) core facility at Oregon National Primate Research Center. The ONPRC assay core has validated the usage of Immulite 2000 for hormones in rhesus monkey including cortisol (Bethea et al., 2005), E_2 and P_4 (Jensen et al., 2008). The Immulite 2000 has been validated independently for monkey ACTH measurements (Vogeser et al., 2000). The sensitivity of estradiol assays is 20 pg/ml, 0.2 ng/ml for progesterone, 10 ng/mL (1 μg/dL) for cortisol, and 5 pg/mL for ACTH. For all hormone assays, values below the level of assay detectability were assigned the minimal detectable concentration in the assay. All quality control samples and validations, provided by the company, were analyzed each time before use for hormonal measurements in samples. The Immulite 2000 runs three QC serum pools daily and thus no specific intra-assay QC data is available. The inter-assay coefficient of variation, reflecting variability in daily QC results over a period of 1.5 years in which these assays were run, was as follows: ACTH 7.7%; cortisol 8.1%; estradiol 8.5%; progesterone 9.4%.

Statistical Analyses

In Experiment 1, a repeated measures ANOVA with post-hoc paired t-test analysis was used to compare baseline levels with the 1-hour post stress levels of ACTH and cortisol in all dosing regimens. The change in ACTH and cortisol levels was calculated as the difference between baseline and post-stress plasma concentrations, and repeated measures contrast analysis was used to test the *a priori* hypothesis that pre-treatment with antalarmin in each of the three doses tested would attenuate the stress-

induced increase in ACTH and cortisol release. Specifically, three pair-wise comparisons were made between the delta value (post stress – baseline) of ACTH and cortisol for the no drug control and each of the three dosing regimens.

There are substantial individual differences in the "normal" frequency of LH bursts from the pituitary in both human and non-human primates (Crowley et al., 1985), which combined with the low sample size of a non-human primate study would lower the statistical power available to detect significant differences using parametric statistics. Therefore, for all analyses of LH pulse frequency in Experiments 2 and 3 Fisher's exact tests were used, assigning a criterion of < or > 4 pulses/8 hours for non-parametric analysis, based on work from Pohl and Knobil (1983) finding a pulse frequency threshold of greater than 3 pulses/8 hours as necessary to induce ovulation in the non-human primate. One HSR and one SS monkey failed to complete Experiment 3. Thus, within-subjects comparisons of data collected in each experimental condition reflect the least common sample size (i.e. n=15 in Experiment 2, n=13 in Experiment 3).

Plasma ACTH and cortisol response to mild combined stress with antalarmin pre-treatment (Experiment 3) were compared to ACTH and cortisol data in Experiments 1 and 4 of Chapter 2 (24-hour ACTH and cortisol in a non-stressed control menstrual cycle and a cycle exposed to mild combined stress, respectively). Although this ACTH and cortisol data is presented in Chapter 2, the samples were collected from the same monkeys using the same procedures as in Chapter 3. Specifically, ACTH and cortisol samples from Chapter 2, Experiment 1 were collected on the same day as LH pulse frequency samples from Chapter 3, Experiment 2, during a non-stressed control menstrual cycle. Likewise, ACTH and cortisol samples from Chapter 2, Experiment 4

were collected on the same day as LH pulse frequency samples from Chapter 3, Experiment 2, during a stressed menstrual cycle. Comparisons of ACTH and cortisol levels in control, stressed, and stress+antalamin conditions were made using a mixed design repeated measures RM-ANOVA (repeated measures design with between-groups comparison). Differences between groups were considered significant if $p \leq 0.05$. All statistical analyses were performed with SPSS version 15.0 statistical software (SPSS Inc, Chicago, IL).

3.3 RESULTS

Six animals were categorized as highly stress-resilient (HSR), five animals were categorized as medium stress-resilient (MSR) and four animals were categorized as stress-sensitive (SS).

Experiment 1. Effects of differential dosing and administration route of antalarmin on ACTH and cortisol responses to acute psychological stress

In response to the psychological stressor of glove exposure, plasma levels of both ACTH and cortisol were significantly elevated as compared to baseline in all drug treatment conditions (Table 3.1; Figure 3.3), indicating that this paradigm is an effective psychological stressor in non-human primates. The baseline measurements of both ACTH and cortisol were indistinguishable between each of the four treatment conditions, even in the oral antalarmin pre-treatment condition that was given prior to collection of the baseline blood sample.

The change in ACTH levels was calculated as the difference between baseline and post-stress concentrations. The only dose and administration route for antalarmin that

resulted in a significant attenuation of ACTH response to the psychological stress was the 3.2 mg/kg dose of intravenous antalarmin (Figure 3.4; Δ $ACTH_{control}$: 67.82±20.55 pg/mL, Δ $ACTH_{3.2\ mg/kg\ i.v.}$: 27.7±10.22 pg/mL; $F_{1,13}$=5.832, p=0.03).

The change in cortisol levels was calculated as the difference between baseline and post-stress concentrations. The only dose and administration route which resulted in a significant attenuation of cortisol response to the psychological stress was the 3.2 mg/kg dose of intravenous antalarmin (Figure 3.5; Δ $cortisol_{control}$: 31.87±3.22 µg/dL, Δ $cortisol_{3.2\ mg/kg\ i.v.}$: 18.88±3.79 µg/dL; $F_{1,13}$=4.975, p=0.04).

Pre-treatment with the higher 10 mg/kg i.v. dose of antalarmin did suppress both ACTH and cortisol levels as compared to the no drug control, although this difference did not quite reach statistical significance (Δ $ACTH_{control}$: 67.82±20.55 pg/mL, Δ $ACTH_{10\ mg/kg\ i.v.}$: 32.12±5.24 pg/mL, p=0.09; Δ $cortisol_{control}$: 31.87±3.22 µg/dL, Δ $cortisol_{10\ mg/kg\ i.v.}$: 25.00±3.58 µg/dL; p=0.14; data not shown).

Experiment 2: Effects of acute stress on LH pulse frequency

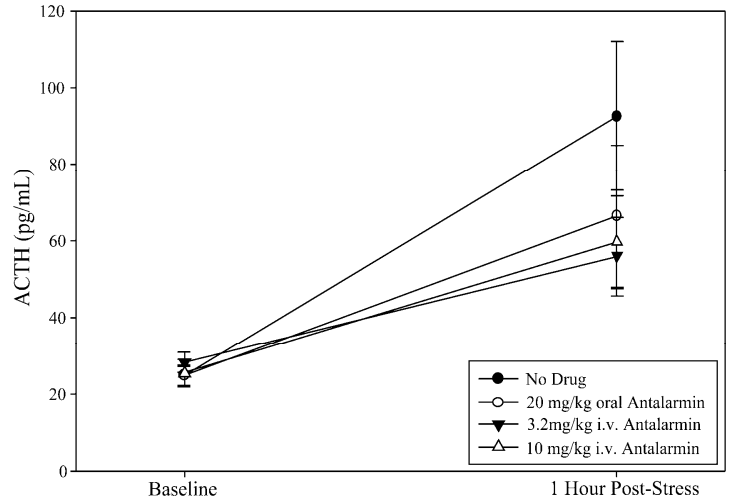
In a normal, non-stressed menstrual cycle, SS monkeys (Figure 3.6; 2.5±1.2 pulses/8 hours) showed a trend toward having lower LH pulse frequency compared to both MSR monkeys (7.6±1.5 pulses/8 hours; p=0.06) and HSR monkeys (7.0±1.8 pulses/8 hours; p=0.08). There were no group differences in either mean LH levels ($F_{2,12}$=0.01, p=0.99) or LH inter-pulse interval ($F_{2,12}$=1.28, p=0.31). HSR monkeys maintained a normal LH pulse frequency in both a control, non-stressed menstrual cycle and in response to acute mild combined stress (Figure 3.7A; Control: 7.00±1.81 pulses/8 hours; Stress: 8.40±1.36 pulses/8 hours; p=0.58). SS monkeys maintained a low pulse

Table 3.1 *Baseline and post-stress plasma ACTH and cortisol concentrations in a no drug control condition and with three different doses and administration routes of antalarmin pre-treatment.*

	ACTH (pg/mg)			Cortisol (µg/dL)		
	Baseline	Post-Stress	p-value*	Baseline	Post-Stress	p-value*
No Drug Control	23.87 ± 3.20	93.34 ± 8.55	p<0.01	23.13 ± 2.72	54.98 ± 3.56	p<0.01
20 mg/kg Oral	24.83 ± 3.29	66.39 ± 8.46	p=0.04	23.32 ± 2.67	48.01 ± 3.73	p<0.01
3.2 mg/kg i.v.	27.10 ± 3.27	55.49 ± 6.18	p<0.01	26.47 ± 2.87	46.54 ± 3.97	p<0.01
10 mg/kg i.v.	25.36 ± 3.67	57.48 ± 6.81	p<0.01	26.88 ± 3.05	51.88 ± 4.11	p<0.01

Values are mean ± SEM. *p-value of paired t-test post-hoc t-statistic comparing baseline to post- every dosing regimen condition.

(A)



(B)

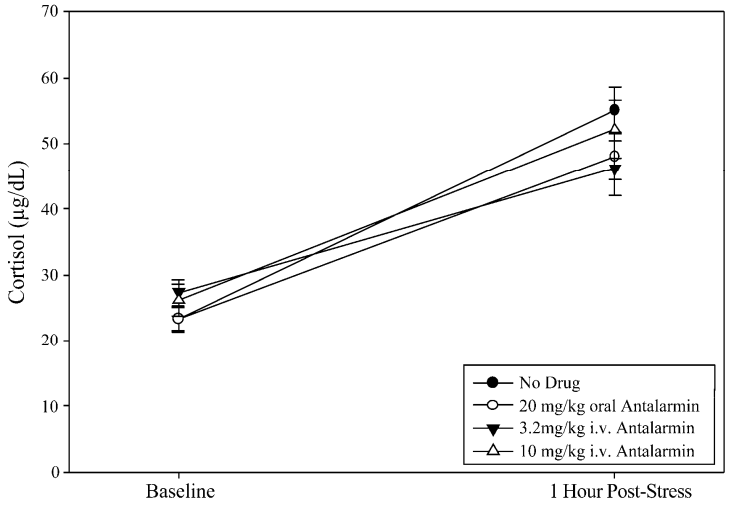


Figure 3.3 Mean concentrations of plasma (A) ACTH and (B) cortisol at baseline and at 1 hr post-stress in all four testing conditions: No Drug (closed circles), 20 mg/kg oral antalarmin (open circles), 3.2 mg/kg i.v. antalarmin (closed triangles), and 10 mg/kg i.v. antalarmin (open triangles). Values are mean ± SEM.

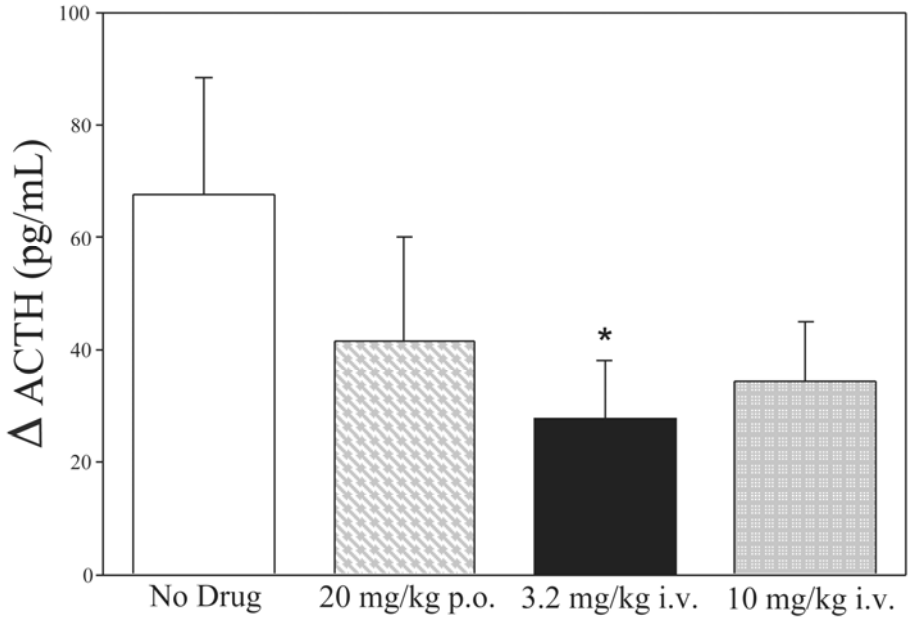


Figure 3.4 Change in plasma ACTH concentration from baseline to one hour post-stress in all four testing conditions. Values are mean ± SEM. Asterisk indicates $p<0.05$, compared to the no drug control.

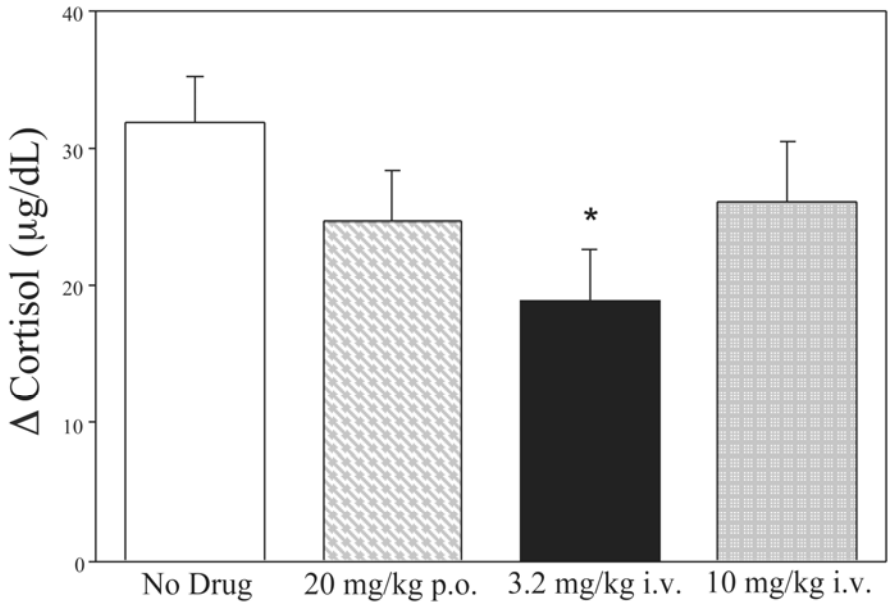


Figure 3.5 Change in plasma cortisol concentration from baseline to one hour post-stress in all four testing conditions. Values are mean ± SEM. Asterisk indicates $p<0.05$ compared to the no drug control.

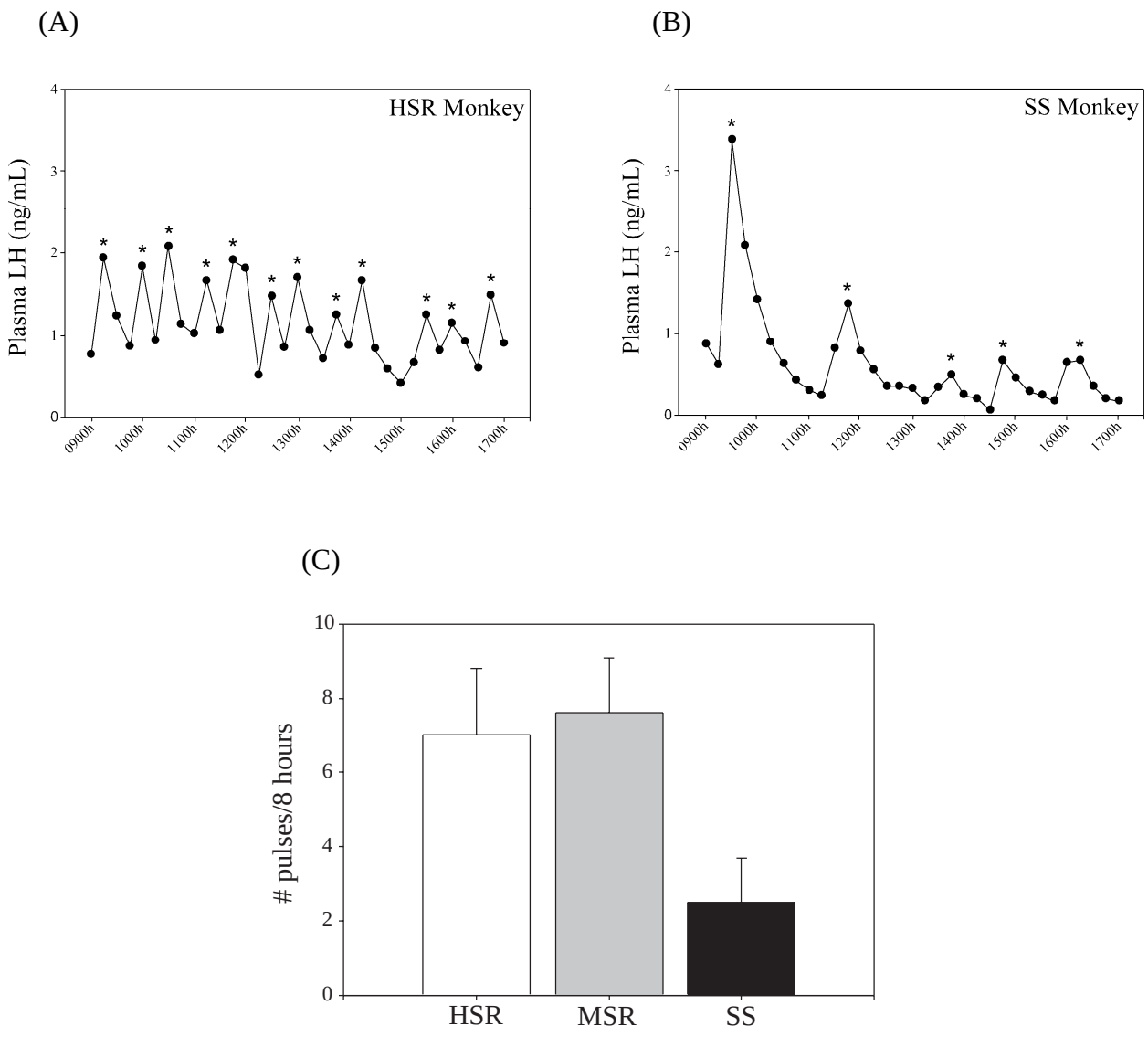


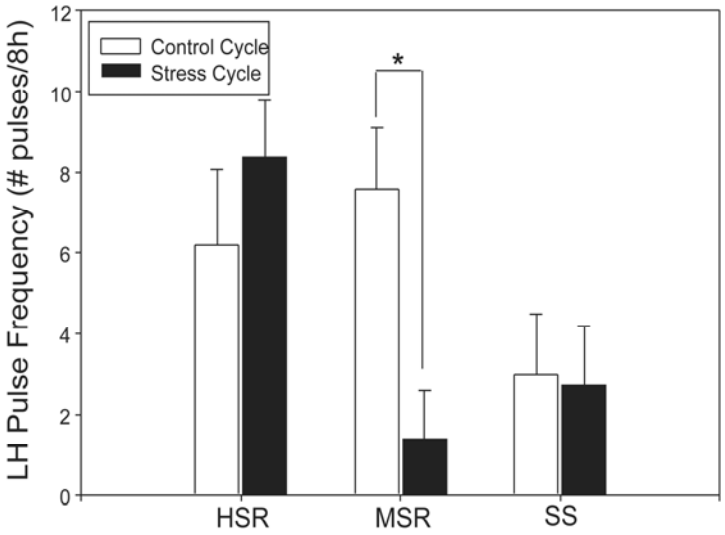
Figure 3.6 Representative LH pulse patterns in one (A) HSR and one (B) SS monkey over an 8-hour period in the early follicular phase of a non-stressed, control menstrual cycle. Asterisks indicate pulses defined by PULSAR algorithm. (C) Mean LH pulse frequency measured in the early follicular phase of a non-stressed, control menstrual cycle in HSR, MSR, and SS monkeys. Values are mean ± SEM.

frequency whether measured in a control, non-stressed menstrual cycle or in response to acute mild combined stress (Figure 3.7A; Control: 2.50±1.29 pulses/8 hours; Stress: 2.00±1.23 pulses/8 hours; p=0.50). However, MSR monkeys showed a relatively high LH pulse frequency in a control cycle (Figure 3.7A; 7.60±1.50 pulses/8 hours) but showed a significant suppression of LH pulse frequency when exposed to acute mild combined stress (1.40±1.19 pulses/8 hours; p=0.02). When animals that became anovulatory in response to mild combined stress (MSR+SS) were grouped, this group showed a significant suppression of LH pulse frequency (Figure 3.7B; Control cycle: 5.33±1.29 pulses/8 hours; Stress cycle: 1.67±0.79 pulses/8 hours; p=0.03), in response to mild combined stress as compared to the control cycle.

Experiment 3: Effect of antalarmin on the HPA axis and LH pulse frequency response to acute stress

Pre-treatment with 10 mg/kg i.v. antalarmin did not suppress ACTH or cortisol in any group, (Figure 3.8; control and stress-only cortisol data taken from Chapter 2, Figure 2.6). In contrast, pre-treatment with antalarmin prior to exposure to mild combined stress prevented the stress-induced suppression of LH pulse frequency in the MSR group (Figure 3.9A; Control cycle: 7.60±1.50 pulses/8 hours; stress + antalarmin cycle: 5.80±2.20 pulses/8 hours; p=0.22), but there was not a similar effect of antalarmin in the HSR or SS groups. Antalarmin also prevented the stress-induced suppression of LH pulse frequency in the combined group of animals that became anovulatory in response to mild

(A)



(B)

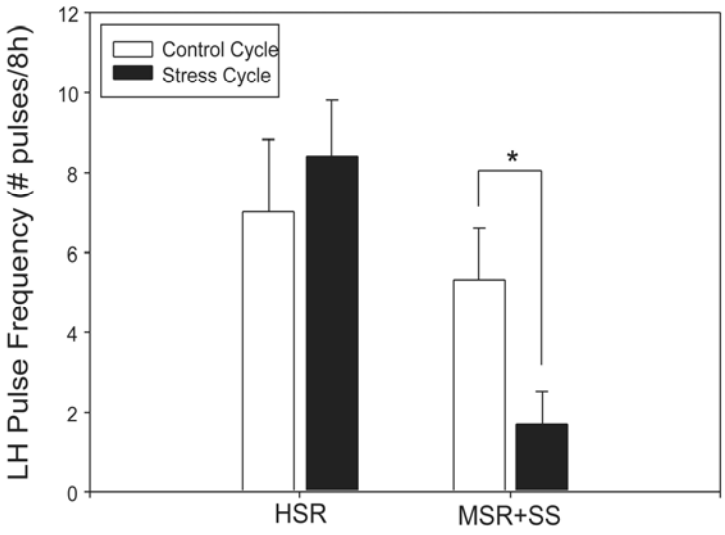


Figure 3.7 LH pulse frequency measured during daytime hours (1000 h to 1800 h) in the early follicular phase of both a control, non-stressed menstrual cycle (open bars) and on the same day in a cycle with mild combined stress (move+diet) in (A) HSR, MSR, and SS monkeys, and (B) in HSR versus animals that became anovulatory in response to mild combined stress (MSR+SS). Values are mean ± SEM. Asterisk indicates $p<0.05$.

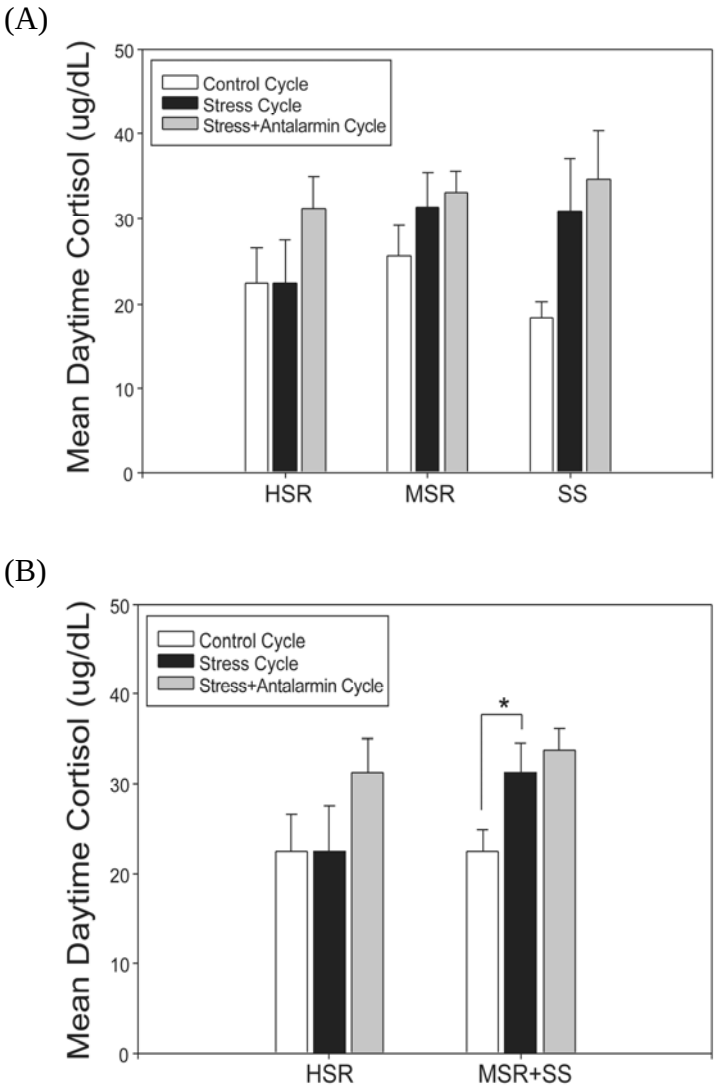
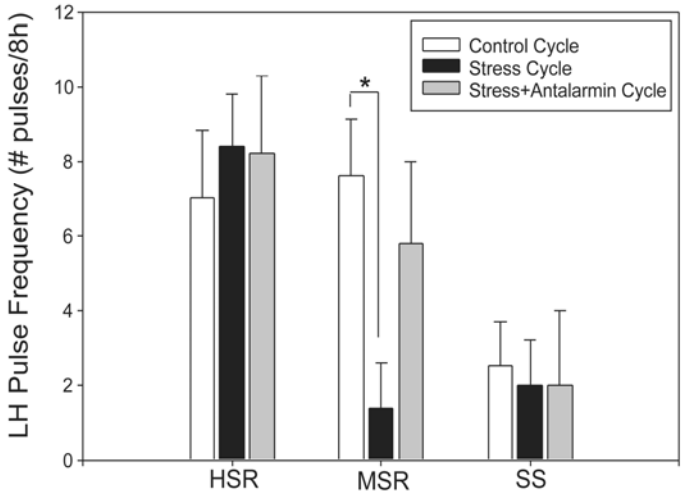


Figure 3.8 Mean levels of plasma cortisol measured during daytime hours (1000 h to 1800 h) in the early follicular phase of both a control, non-stressed menstrual cycle (open bars) and in the same phase of a cycle with (gray bars) and without (black bars) antalarmin pretreatment prior to exposure to mild combined stress (move+diet) in (A) HSR, MSR, and SS monkeys, and in (B) in HSR versus animals that became anovulatory in response to mild combined stress (MSR+SS). Control and stress cycle data reprinted from Figure 2.6. Values are mean ± SEM. Asterisk indicates $p<0.05$.

(A)



(B)

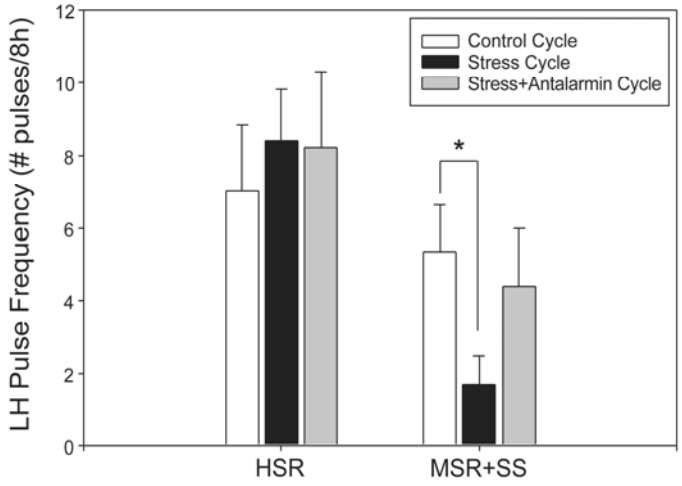


Figure 3.9 LH pulse frequency measured during daytime hours (1000h to 1800h) in the early follicular phase of both a control, non-stressed menstrual cycle (open bars) and on the same day in a cycle with (gray bars) and without (black bars) pre-treatment with antalarmin prior to exposure to mild combined stress (move+diet) in (A) HSR, MSR, and SS monkeys, and (B) in HSR versus animals that became anovulatory in response to mild combined stress (MSR+SS). Values are mean ± SEM. Asterisk indicates $p<0.05$.

combined stress (MSR+SS; Figure 3.9B; Control cycle: 5.33±1.29 pulses/8 hours; stress + antalarmin cycle: 4.38±1.63 pulses/8 hours; p=0.25).

3.4 DISCUSSION

In this study, I report that in a non-stressed, control menstrual cycle, stress-sensitive monkeys that readily develop stress-induced amenorrhea have decreased LH pulse frequency when compared to more stress-resilient animals, indicating that these individuals have decreased central drive to the reproductive axis even in the absence of overt stress. I also found that there is an acute effect of stress on the reproductive axis, as measured by acute suppression of LH pulse frequency following exposure to mild psychosocial and metabolic stress in some, but not all, monkeys. HSR animals showed no suppression of pulsatile LH secretion when exposed to acute stress. In contrast, acute exposure to mild combined stress caused MSR monkeys to suppress the frequency of pulsatile LH secretion to levels typical of SS monkeys. Interestingly, acute stress exposure did not lead to a further suppression of LH pulse frequency in SS monkeys that show LH secretory impairment even in non-stressed conditions. And when considered together, monkeys in which stress can lead to anovulation (i.e., MSR+SS animals) showed a relatively high frequency of pulsatile LH secretion in control conditions but showed a significant impairment of pulsatile LH secretion when acutely stressed. Importantly, pre-treatment with the specific CRH-R1 antagonist, antalarmin, prior to exposure to mild combined stress was able to prevent acute stress-induced suppression of LH pulse secretion *without* suppressing activity of the HPA axis in MSR+SS animals.

Because antalarmin prevented a stress-induced suppression of LH pulse frequency, without preventing stress-induced suppression of ACTH or cortisol secretion, these findings suggest that reproductive effects of antalarmin are likely to act through non-neuroendocrine mechanisms (i.e., not through suppression of the HPA axis) to regulate other neurotransmitter systems that modulate the activity of the reproductive axis.

The reported effects of antalarmin on reproductive function have been extremely limited. Antalarmin infusion into the amniotic sac, fetus, and jugular vein of pregnant ewes can delay pre-term birth onset by suppressing the increase in fetal CRH normally associated with triggering parturition (Chan et al., 1998). Interestingly, similar to our findings in this study, maternal cortisol did not differ between vehicle- and antalarmin-treated ewes in that study. The reproductive effects of antalarmin may not, however, be all beneficial. Makrigiannakis and colleagues (2001) reported that female rats treated with antalarmin showed a 70% decrease in implanted embryos in early pregnancy. This increase in early miscarriage with CRH-R1 receptor antagonism is thought to occur by inhibiting the apoptosis of activated T-lymphocytes, a process which is normally induced by CRH in the uterus, thus protecting the fetus from T-lymphocyte attack and maintaining early pregnancy (Kalantaridou et al., 2004). While I report here that treatment with antalarmin is able to restore LH pulse frequency following stress exposure, the full reproductive effects at each level of the HPG axis and resulting outcomes for successful menstrual cyclicity, fertility, establishment and maintenance of pregnancy remain to be fully studied.

In Chapter 2 of this dissertation, I found that animals that become anovulatory in response to stress (MSR+SS) have an increase in daytime cortisol secretion following a

move to novel room plus reduced diet. In Chapter 3, Experiment 3, I found that treatment with antalarmin did not suppress the stress-induced increase in daytime cortisol reported in Chapter 2. This finding was quite unexpected given that data from Chapter 3, Experiment 1 indicated that, while perhaps not as effective as a lower dose of antalarmin (i.e., 3.2 mg/kg), intravenously administered antalarmin at a dose of 10 mg/kg, i.v., did show a trend towards suppressing both ACTH and cortisol in an acute psychological stress paradigm. It is likely that the different stressors used (exposure to a leather catch glove was used in Chapter 3, Experiment 1 vs. mild diet + move to novel room was used in Chapter 3, Experiment 3) made a substantial difference in the effects of antalarmin on ACTH and cortisol secretion. In the case of the pilot study, leather catch glove exposure was designed to imitate hand-catching. This is a significant stressor and was intentionally used in order to provide a condition in which it would be possible to detect suppression of stress-induced ACTH and cortisol by antalarmin. However, the combined stress of reduced diet + move to a novel room used in Chapter 3, Experiment 3 is a much milder stressor, and may have contributed to the differential findings.

The reported effects of antalarmin on HPA axis activation and stress-induced behaviors in rodents and primates are inconsistent. The finding that the 3.2 mg/kg i.v. dose, but not the higher 10 mg/kg i.v. dose, suppresses the ACTH and cortisol response to psychological stress is similar to previous studies by Broadbear and colleagues (2004) who reported similar results using a CRH challenge, rather than psychological stress, to activate the pituitary-adrenal axis. However, Broadbear et al. (2004) reported that the 10 mg/kg i.v. dose of antalamin increased cortisol response to CRH. Unlike the Broadbear studies I did not find that the 10 mg/kg i.v. dose of antalarmin increased the cortisol

response to stress when compared to cortisol response to vehicle. The oral administration of antalarmin (20 mg/kg) did not significantly attenuate ACTH or cortisol activation to psychological stress, which is consistent with some (Deak et al., 1999; Wong et al., 1999; Ayala et al., 2004; Myers et al., 2005), but not all (Webster et al., 1996; Bornstein et al., 1998; Habib et al., 2000; French et al., 2007) previous studies in rodents and primates utilizing this dose and oral administration. The reasons for this discrepancy are unknown, as the preparation, administration and experimental time course were modeled after a previous study reporting significant attenuation of HPA axis activation and stress-related behavior with oral antalarmin (Habib et al., 2000). The 20 mg/kg oral dose may not have suppressed either stress-induced ACTH or cortisol because not enough orally-administered antalarmin may have penetrated the blood-brain barrier. The bioavailability of orally-administered drugs is much lower than that of intravenous drugs because they pass through the liver immediately after absorption and are subjected to degradatory enzymes before reaching their target sites in the brain and pituitary. It is also possible that the different behavioral stress paradigms or psychological stress versus CRH or immune challenge are differentially impacted by antalarmin. A species differences could also influence the response to or clearance of this drug.

Similarly, there are several possible reasons that may explain why stress-induced activation of ACTH and cortisol were not attenuated by the 10 mg/kg i.v. dose antalarmin. Broadbear and colleagues (2004) reported that pre-treatment with 10 mg/kg i.v. antalarmin did not suppress ACTH, but actually stimulated cortisol release in response to a CRH challenge. This finding suggests that at higher intravenous doses, this selective CRH-R1 antagonist may also have partial agonistic effects on the CRH-R1

receptor. Although I did not find a stimulation of stress-induced cortisol release with the 10 mg/kg, i.v. dose of antalarmin, I did find that this higher dose of antalarmin was less effective in suppressing stress-induced cortisol than the 3.2 mg/kg i.v. dose. The use of the higher 10 mg/kg i.v. dose of antalarmin in Chapter 3, Experiment 3 may not have been the most effective in suppressing ACTH and cortisol response to acute mild combined stress. However, the finding that antalarmin was able to partially prevent the stress-induced suppression of LH pulse frequency in spite of a lack of an effect on either stress-induced ACTH or cortisol release, indicates that the increased cortisol response to mild combined stress observed in MSR+SS monkeys (Chapter 2) is not causal to the acute stress-induced suppression of LH pulse frequency in these same monkeys.

It is also interesting that female monkeys with different sensitivities to stress-induced reproductive dysfunction had differences in frequency of pulsatile LH secretion in a non-stressed condition, as well as different responses of the central pulse generator to stress. Monkeys that are resilient to stress-induced reproductive dysfunction have a LH pulse frequency typical of the frequency that occurs in the early follicular phase (i.e., approximately one pulse per hour; Pohl & Knobil, 1982). In HSR monkeys the circhoral release of LH was maintained even in response to acute mild combined stress. In contrast, SS monkeys had a slower LH pulse frequency, and therefore presumably decreased central GnRH drive to the reproductive axis, even in the absence of overt stress. As expected, SS monkeys that had fewer than 3 pulses in the 8-hour sampling period (i.e., 2 of the 4 SS monkeys) did not ovulate in the study cycle. This is in agreement with previous literature finding that a minimum of 3 pulses in an 8-hour period is necessary to induce ovulation in female monkeys (Pohl et al., 1983). Interestingly,

exposure to acute mild combined stress did not further suppress LH pulse frequency in SS animals, possibly due to an already compromised GnRH pulse generator operating sub-optimally in normal, non-stressed conditions. MSR monkeys, that only lose menstrual cyclicity in response to prolonged exposure to stress, had a circhoral LH pulse frequency in normal, non-stressed conditions but rapidly suppressed the frequency of pulsatile LH secretion in response to acute mild combined stress. This stress-induced suppression of pulsatile LH could reflect increased inhibitory input to the reproductive axis via increased GABA, CRH, or opioidergic mechanisms, decreased stimulatory input through serotonin or glutamatergic mechanisms, or both, as differences in several of these neurotransmitter systems have been reported in stress-sensitive monkeys (Bethea et al., 2005a,b; Centeno et al., 2007a,b).

Previous work utilizing this animal model has characterized the secretion of reproductive steroid hormones in stress-sensitive versus stress-resilient monkeys and reported similar findings to what has been observed in women with stress-induced amenorrhea versus normally cycling women (Reame et al., 1985; Bethea et al., 2008). Monkeys that are highly resilient to stress-induced reproductive dysfunction, like eumenorrheic women, develop normal pre-ovulatory levels of circulating estradiol, even during menstrual cycles in which they were exposed to everyday life stresses (Bethea et al., 2008). My current finding that these highly stress-resilient monkeys have normal LH pulse frequency even in response to acute mild combined stress corroborates this, and more fully characterizes the response of the hypothalamic-pituitary-gonadal (HPG) axis to stress as being highly resilient in these individuals. Previous studies in the laboratory also have shown that stress-sensitive monkeys have lower pre-ovulatory levels of

estradiol that rapidly suppress in response to mild combined stress, resulting in a loss of menstrual cyclicity (Bethea et al., 2008). My finding that individuals sensitive to stress-induced reproductive dysfunction have decreased central drive to the HPG axis even in the absence of overt stress may account for the lower estradiol secretion. In this study I did not find that exposure to everyday life stresses further suppresses LH pulse frequency in these monkeys, which I attribute to the already slow pulsatile secretion of LH in normal conditions. However, it is possible that in a larger sample of stress-sensitive monkeys a further suppression of LH secretion might be observed if the majority of animals completely suppressed LH secretion to non-detectable levels when exposed to stress.

MSR monkeys, that have been defined in this study and in previous studies by our laboratory as losing reproductive function only in response to prolonged exposure to everyday life stress (Bethea et al., 2008), have a LH pulse pattern that resembles HSR monkeys in a normal, non-stressed menstrual cycle, and SS monkeys in a menstrual cycle in which they were exposed to acute mild combined stress. This characterization of pituitary secretion of LH in control and stressed menstrual cycles also supports previously observed steroid hormone activity in MSR individuals (Bethea et al., 2008). Medium stress-resilient monkeys typically have peak estradiol and progesterone levels comparable to those of highly stress-resilient monkeys in normal, non-stressed conditions, as well as in response to short-term (one menstrual cycle's length) exposure to mild combined stress. However, with repeated exposure to mild combined stresses (i.e., in a second menstrual cycle), they suppress secretion of both estradiol and progesterone to levels similar to what is observed in stress-sensitive monkeys. My

finding that the medium stress-resilient monkeys have normal LH pulse frequency in a control, non-stressed follicular phase but rapidly suppress LH secretion to frequencies similar to that of stress-sensitive monkeys corroborates these previous findings. It may be that medium stress-resilient monkeys are acutely affected in the early follicular phase by everyday life stresses but are able to recover throughout the remainder of the cycle to ovulate and have a normal cycle, unless this stress is prolonged. This acute response to mild combined stress and subsequent recovery is in contrast to that of stress-sensitive animals that appear to not be able to recover from the effects of everyday life stresses and fail to have an ovulatory menstrual cycle.

The findings reported herein further support the validity of this non-human primate model of stress-induced reproductive dysfunction by indicating that reproductive disruption in stress-sensitive monkeys occurs at least at the level of the pituitary. Furthermore, while I did report in Chapter 2 that MSR and SS monkeys show an elevation of daytime cortisol in response to mild combined stress, but not in baseline non-stressed conditions, my finding in this study that treatment with a specific CRH-R1 antagonist is able to prevent the acute stress-induced suppression of LH pulse frequency without blocking the previously reported increase in daytime cortisol following stress further indicates that activation of the HPA axis is not a primary neural mechanism underlying sensitivity to stress-induced reproductive dysfunction.

Chapter 4

INCREASED CORTICOTROPIN-RELEASING HORMONE INPUT INTO THE RAPHE NUCLEUS IN FEMALE MONKEYS SENSITIVE TO STRESS-INDUCED REPRODUCTIVE DYSFUNCTION

4.1 INTRODUCTION

Both women and female monkeys can show a suppression of normal function of the reproductive axis in response to everyday life stresses (Cameron, 1997; Cameron, 2000; Marcus et al., 2001; Bomba et al., 2007). Clinically, one of the most common forms of stress-induced reproductive function is stress-induced amenorrhea, a disorder thought to be caused by a combined effect of mild psychosocial stress + metabolic stress, acting to suppress GnRH drive to the reproductive axis and inhibit normal reproductive function (Marcus et al., 2001; Giles & Berga, 1993; Berga et al., 1997). In women with stress-induced amenorrhea elevated cortisol levels have been observed in some (Biller et al., 1990; Berga et al., 1997; Meczakalski et al., 2000; Kondoh et al., 2001; Brundu et al., 2006), but not all (Couzinet et al., 1999) studies, indicating a possible mechanistic role for hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis in this disorder.

Our laboratory has shown that female monkeys differ in the sensitivity to stress-induced reproductive dysfunction, such that some individuals rapidly become anovulatory in response to stress ("stress-sensitive"; SS), while others are more stress-resilient (Cameron, 1997; Cameron, 1998; Bethea et al., 2005a; Bethea et al., 2005b; Centeno et al., 2007a; Centeno et al., 2007b; Williams et al., 2007; Bethea et al., 2008). Interestingly, our laboratories have previously reported that stress-sensitive monkeys

have increased corticotropin-releasing hormone (CRH) expression in the paraventricular nucleus (PVN) of the hypothalamus, amygdala and thalamus, as well as decreased physiological release of serotonin and decreased expression of several serotonin-related genes as compared to highly stress-resilient monkeys (Bethea et al., 2005a,b; Centeno et al., 2007a). However, studies in Chapter 2 of this dissertation showed that SS monkeys do not differ from more stress-resilient animals in five different measures of HPA axis activity, and studies in Chapter 3 showed that pre-treatment with a CRH-R1 antagonist prevented the stress-induced suppression of LH pulse frequency without preventing the stress-induced increase in cortisol secretion. Taken together, these data indicate that greater activity of the HPA axis is not the primary mechanism underlying the development of sensitivity to stress-induced reproductive dysfunction in this monkey model. CRH is the neurohormone released from the paraventricular nucleus (PVN) of the hypothalamus that regulates activation of the HPA axis in response to stress (Vale et al., 1981). However, CRH is also a neurotransmitter that can act directly or in concert with other neurotransmitter systems to regulate autonomic and behavioral responses to stress (Dunn & Berridge, 1990; Valentino et al., 1993). It may be that the elevation in CRH gene expression that is apparent in stress-sensitive (SS) monkeys plays a role in suppressing the reproductive axis by acting as a neurotransmitter rather than by acting as the neuroendocrine regulator of the HPA axis.

One neural system regulated by CRH acting as a neurotransmitter is the network of serotonergic neurons in the dorsal and median raphe nucleus (Cole & Sawchenko, 2002). Both CRH-immunoreactive fibers (Swanson et al., 1983; Sakanaka et al., 1987), as well as mRNA for the two known CRH receptors (Potter et al., 1994; Chalmers et al.,

1995), have been localized to serotonergic neurons in these two nuclei. The effects of CRH on serotonin neuronal firing have been reported to be largely inhibitory (Price et al., 1998; Kirby et al., 2000; Price & Lucki, 2001). Intracerebroventricular administration of CRH has been shown to inhibit concentrations of serotonin in microdialysate and the spontaneous discharge rate of neurons in the dorsal raphe (Price et al., 1998). Administration of CRH into the dorsal raphe nucleus (DRN) produces a learned helplessness behavior in rodents, which has long been associated with low serotonin levels (Hammack et al., 2002, 2003). However, ultrastructural studies show both symmetrical (i.e. inhibitory) and asymmetrical (i.e. excitatory) synapses between CRH-immunoreactive terminals and serotonergic dendrites (Valentino et al., 2001), with topographic organization. Specifically, while a majority of these connections are inhibitory, a small subpopulation of serotonergic neurons in the dorsolateral DRN are electrophysiologically activated, rather than suppressed, in response to CRH administration (Lowry et al., 2000).

As serotonin can play a modulatory role in regulating the reproductive axis (Tilbrook et al., 2001; Dobson et al., 2003), specifically having been shown to stimulate gonadotropin-releasing hormone (GnRH) neuronal activity in the rat (Smith and Jennes, 2001), it is possible that CRH-induced suppression of serotonin is an underlying neural mechanism for reproductive dysfunction in stress-sensitive monkeys. In this study I quantified immunoreactive CRH fibers in the dorsal and median raphe nuclei of monkeys with different sensitivities to stress-induced reproductive dysfunction to begin to test the hypothesis that stress-sensitive monkeys have lower serotonergic neuronal activity, in part as a result of increased CRH neuronal input into the raphe nucleus.

4.2 MATERIALS AND METHODS

Animals

Ten adult female cynomolgus monkeys (*Macaca fascicularis*) were housed in single cages in a temperature controlled room (24 ± 2°C) with lights on 12 hours a day (0700 h to 1900 h). At the time of tissue collection, the monkeys were 12-13 years of age, as established by dental aging when they were 2-3 years of age (Clifton et al., 1982). Monkeys were fed two meals a day at 0900 h and 1530 h consisting of six high protein monkey chow biscuits (Purina Monkey Chow #5045, Ralston Purina Co., St. Louis, MO; approximately 308 kcal/meal), and one-quarter piece fresh fruit with the afternoon meal. Water was available *ad libitum*. Each animal had their vaginal area swabbed daily to check for menses. The first day of menstrual bleeding was designated Day 1 of a menstrual cycle. Food intake was measured at each meal and weight was measured weekly. All protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center.

Characterization of Stress Sensitivity

Each monkey had been tested for the sensitivity of the reproductive axis to stress by assessing the response of the reproductive axis in terms of ovulation and reproductive hormone secretion when animals were exposed to a mild combined stress which consisted of mild psychosocial stress + moderate metabolic stress (diet and exercise) (Williams et al. 2007). The mild psychosocial stress used in this study involved moving monkeys housed in a single cage to a novel room, where they were surrounded by unfamiliar monkeys. The metabolic stress consisted of a reduction in calorie intake (20%

reduction in available calories) + moderate exercise [running monkeys on a motorized treadmill at 80% maximum heart rate (as determined for each animal in the first week of the study) for one hour a day, 5 days per week].

Characterization of stress-sensitivity involved a five menstrual cycle design (Chapter 1, Figure 1.3; Williams et al. 2007). Cycle 1 was a control cycle in which blood samples were collected daily to monitor baseline concentrations of reproductive hormones throughout a normal menstrual cycle. In Cycle 2, animals were trained to run on a motorized treadmill by first sitting on it and watching other monkeys run and then walking. Cycle 3 was Stress Cycle I, during which monkeys were moved to a novel room on the first day of the menstrual cycle, available caloric intake was decreased by 20% and animals were exercised (with the rate of running and daily duration of running increasing progressively until they were running at 80% maximal capacity for 1 hr/day, 5 days/wk). Cycle 4 was Stress Cycle 2, during which monkeys were again moved on Day 1 of their menstrual cycle to a single cage in a different novel room and calorie restriction and exercise training continued. In Cycle 5 monkeys were allowed to recover from the stress condition by returning to their home environment, with caloric intake increased to baseline levels and exercise training terminated. Monkeys were classified as highly stress-resilient (HSR; n=4) if they maintained two normal ovulatory menstrual cycles throughout both stress cycles. Monkeys were classified as stress-sensitive (SS; n=4) if they became anovulatory immediately upon exposure to the mild combined stress paradigm. Two monkeys were categorized as medium stress-resilient (MSR) but tissue from these animals was not available for the purposes of this study.

Euthanasia and Tissue Preparation

All animals were characterized for stress-sensitivity in 1998 to 2000, as described previously (Bethea et al., 2005a,b; Williams et al., 2007; Bethea et al., 2008). They were characterized for peak follicular phase estradiol and peak luteal phase progesterone (Bethea, Centeno, & Cameron, 2008) and physiological response to fenfluramine and CRH challenge (Bethea et al., 2005a) in 2001 and then maintained under normal, non-stressed conditions (full diet and no treadmill training) and housed in their home cages for several years. At the end of their experimental protocols, upon detection of menstruation, the animals were scheduled for euthanasia in the early follicular phase (Day 1-5) of their menstrual cycle. Thus, approximately three years after stress-sensitivity assessment and under control, non-stressed conditions, the monkeys were euthanized according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Molecular grade chemicals and solvents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Each animal was sedated with Ketamine hydrochloride (10 mg/kg i.v.) in their home cage, transported to the necropsy suite, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta. The left ventricle of the heart was cannulated and the head of each animal was perfused with 1 liter of saline followed by 7 liters of 4% paraformaldehyde in 3.8% borate buffer, pH 9.5. The brain was removed and cut into tissue blocks (Sanchez et al., 2005). Tissue blocks were post-fixed in 4% paraformaldehyde for 3 hours, then transferred to 0.02 M potassium phosphate-buffered saline (KPBS) containing 10% glycerol for 24 h, followed by 20% glycerol and 2% dimethyl sulfoxide (DMSO) for 48 h at 4°C to cryoprotect the tissue. After glycerol

infiltration was complete, the block was frozen in isopentane cooled to –55°C, and stored at –80°C until sectioning. The pontine midbrain containing the dorsal and median raphe nuclei was sectioned in the coronal plane at 25 µm intervals. The sections were allocated to sets that contained a rostral to caudal representation of the dorsal and median raphe at 250 µm intervals. Eight sections (250 µm apart) containing the fullest extent of the dorsal and median raphe were placed in individual wells containing a cryoprotectant buffer (30:20:50, ethylene glycol:glycerol:0.05M $NaHPO_4$) at -20°C until processing for immunocytochemistry. For this study, four alternating sections each at 500µm apart, spanning the dorsal and median raphe were used.

Immunocytochemistry

Sections were removed from the -20°C freezer and washed in 0.02 M KPBS buffer 4 times for 15 min each, followed by a 30 minute incubation in 0.6% H_2O_2 in 0.02 M KPBS and another 4x15 minute wash series in 0.02 M KPBS. Sections were then blocked against non-specific staining for 1 hour with 2% NGS (normal goat serum) in 0.2 M KPBS + 0.4% Triton for better signal specificity, then incubated with Vector ABC kit reagents Avidin and Biotin in 0.02 M KPBS buffer for 20 minutes each, and in 1% human α-globulin in 0.02 M KPBS buffer for 30 minutes. Sections were then incubated for 48 hours with CRH antibody (gift of Dr. Wylie Vale, Salk Institute, La Jolla, CA) in 2% NGS/10% human α-globulin/0.2 M KPBS + 0.4% Triton solution at 4°C. The antiserum to CRH has been extensively characterized and previously applied to primate brain (Palkovits et al., 1985; Bassett and Foote, 1992). Pilot studies on other sections were undertaken to confirm the optimal antibody dilution (i.e. the lowest dilution yielding

as little background as possible with good signal specificity) as 1:15,000 for these experiments. A negative control section was processed with experimental sections by omitting primary antibody, and no visible staining was observed in this section. Sections were then washed for 4x15 minutes with 0.02 M KPBS buffer and incubated in Vector biotinylated goat anti-rabbit serum in 0.2 M KPBS + 0.4% Triton for 60 minutes. Sections were then incubated in Vector ABC reagent in 0.2 M KPBS + 0.4% Triton for 60 minutes, and then incubated with 3,3'-diaminobenzidine (DAB; in 0.02 M KPBS plus 3% H_2O_2) for 20 minutes, undergoing 4x15 minute serial washes with 0.02 M KPBS buffer between each incubation. Finally, sections were dehydrated through a graded series of ethanols, xylene and Histoclear and mounted under glass with 1,3-diethyl-8-phenylxanthine (DPX).

Stereological Analysis of Immunostaining

Sections were anatomically matched between animals using three anatomical reference points, and a Marianas stereological workstation with Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO) was used for analysis. Each section was examined entering guidepoints demarcating the area of the dorsal raphe nucleus (DRN). The workstation captures multiple 10x images across the span of the DRN and constructs a montage across the entire area of the DRN, for each of the 4 sections through the DRN of each animal. The CRH-positive neuronal fibers were highlighted and the area covered by highlight was computed as positive pixel area in both pixels and microns, allowing limitations such as contrast and removal of background signal from analysis. Due to poor

mounting of two out of four sections that resulted in folded tissue over the area containing the dorsal raphe, one SS animal had to be excluded from analysis.

Statistics

The CRH-positive fiber staining signals were measured as positive pixel area (μm^2). The measurements from each section containing the dorsal raphe nucleus were compared at each level (alternating 250 μm sections; Levels 1-4 spanning the DRN) between groups. The positive pixel area at each level was then averaged to produce one value for the entire region of the DRN for each animal, representing average immunostained area in microns. A mixed design repeated measures ANOVA (RM-ANOVA) was used to test interaction and main effects of Bregma level (rostral to caudal levels 1-4 throughout both dorsal and median raphe nucleus) and stress-sensitivity group.

4.3 RESULTS

CRH staining was robust in the DRN of SS animals and by contrast was decreased to nearly undetectable levels in the HSR animals, with very little observable background staining (Figure 4.1). Data obtained from positive pixel staining from both the dorsal and median raphe nucleus met assumptions for sphericity and as such, no epsilon correction was made. In the dorsal raphe nucleus (DRN), RM-ANOVA failed to detect an interaction effect of level by stress-sensitivity group ($F_{3,15}$=0.75, p=0.54), while there was a significant main effect of level ($F_{3,15}$=14.51, p=0.00) due to neuroanatomical volumetric changes in the rostral to caudal plane. There was also a main effect of stress-

sensitivity group, as the immunostained area of CRH fibers (Figure 4.2) was greater in the dorsal raphe nucleus of SS monkeys (116,785 ± 12,734 μm^2) as compared to HSR monkeys (64,083 ± 5,670 μm^2; $F_{3,15}$=17.52, p=0.01). In the median raphe nucleus (MRN), RM-ANOVA failed to detect an interaction effect of level by stress-sensitivity group ($F_{3,15}$=2.18, p=0.14), although like in the DRN, there was also a main effect of level ($F_{3,15}$=27.02, p=0.00). There was also a main effect of stress-sensitivity group in the MRN. The immunostained area was increased in the median raphe nucleus of SS monkeys (89,225 ± 8,632 μm^2) as compared to HSR animals (57,842 ± 7,347 μm^2; $F_{3,15}$=7.71, p=0.04, Figure 4.3).

4.4 DISCUSSION

In this study, I present evidence that monkeys sensitive to stress-induced reproductive dysfunction (i.e. monkeys that immediately become anovulatory following exposure to mild psychosocial + metabolic stress) have more CRH immunopositive fiber staining in the dorsal and median raphe nuclei as compared to more stress-resilient monkeys. This suggests that CRH neurons may be acting in a non-neuroendocrine manner to contribute to the suppression of reproductive function in response to everyday life stresses in stress-sensitive female cynomolgus monkeys.

Serotonin mediates many aspects of both behavioral and physiological responses to stress, particularly in cases of chronic or severe psychosocial stresses (for review see Holmes, 2008). Importantly, the serotonin system has been extensively linked to many psychosocial stress-induced depression and anxiety disorders (Lowry et al., 2005; Tyrer

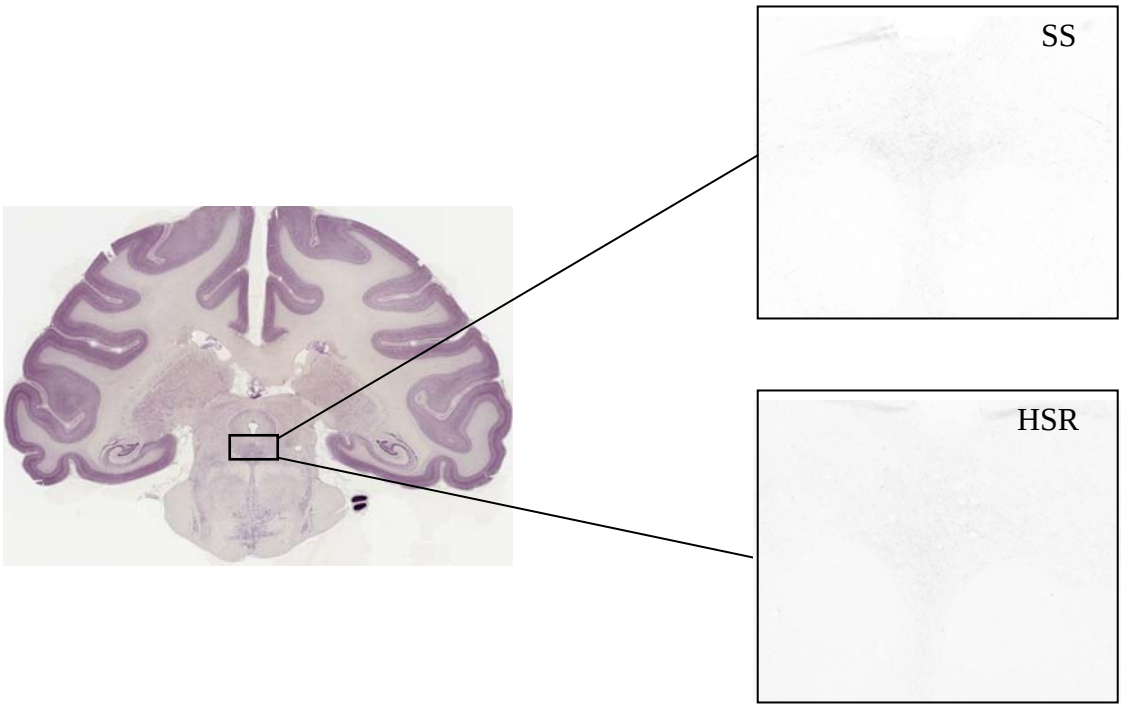


Figure 4.1 Representative staining of the dorsal raphe nucleus from one highly stress-resilient (HSR) and one stress-sensitive (SS) monkey. Immunocytochemistry was performed using CRH antibody (gift from Dr.. Wylie Vale, Salk Institute, La Jolla, CA) at four levels extending the length of the dorsal raphe nucleus, 500 μm apart, from 4 HSR and 3 SS monkeys. Coronal intact brain image courtesy of http://www.brainmaps.org.

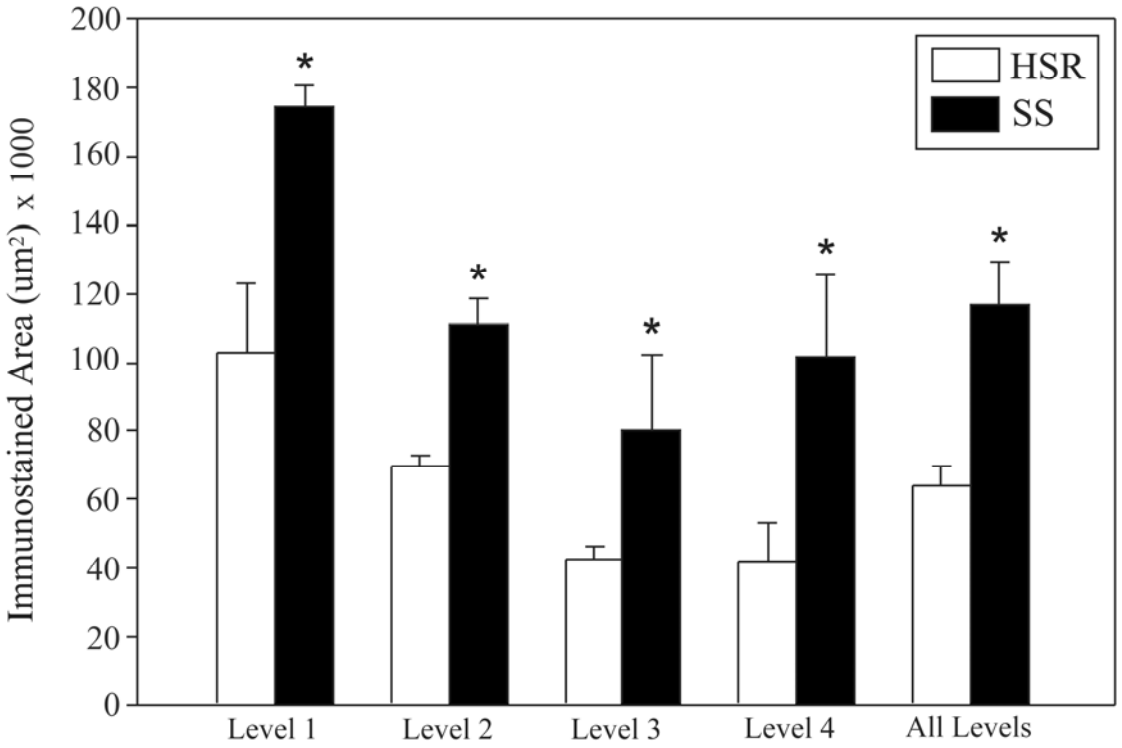


Figure 4.2 Immunostained area (μm^2) of CRH fibers in the dorsal raphe nucleus of highly stress-resilient (open bars) and stress-sensitive (closed bars) monkeys. Values are mean ± SEM. Asterisks indicate $p<0.05$.

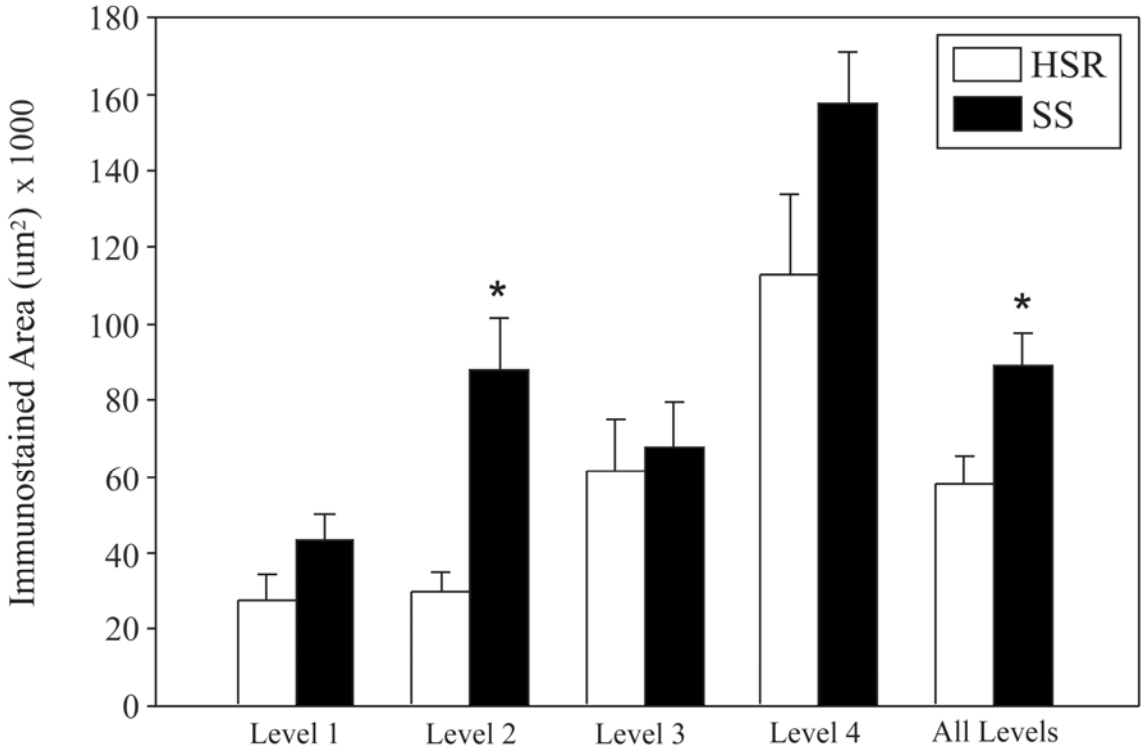


Figure 4.3 Immunostained area (μm^2) of CRH fibers in the median raphe nucleus of highly stress-resilient (open bars) and stress-sensitive (closed bars) monkeys. Values are mean ± SEM. Asterisks indicate $p<0.05$.

& Baldwin, 2006), which have been documented in up to 50% of women with stress-induced amenorrhea (Fava et al., 1984). Currently, the most widely utilized first line treatment for depression and anxiety disorders are selective serotonin reuptake inhibitors (SSRIs) (Goodman, 2004), and association studies have shown functional polymorphisms in both the serotonin transporter (5-HTT) and monoamine oxidase A (MAO-A) gene promoter regions in individuals with anxiety disorders (Greenberg et al., 2000; Murakami et al., 1999; Samochowiec et al., 2004). It has also been well documented that serotonin neurons, in a balance with other neurotransmitters and neuropeptides, regulate the activity of the hypothalamic-pituitary-gonadal (HPG) axis by maintaining GnRH neuronal activity (Dobson et al., 2003; Tilbrook et al., 2002; Smith et al., 2003; von Borell et al., 2007). In the rat, blockade of serotonin biosynthesis or lesioning of the dorsal and median raphe nuclei results in suppression of LH pulse frequency or complete blockade of the LH surge, respectively (Hery et al., 1976, 1978). This is thought to occur at the level of the hypothalamus, as serotonergic neurons from the raphe nuclei project to the mediobasal hypothalamus and increase GnRH secretion in portal blood (Smith and Jennes, 2001). While there is less known regarding the specific mechanism of serotonin-mediated HPG axis regulation in primates, the distribution of serotonergic neurons is highly conserved among most species (Tillet, 1995) and as such serotonin neurons are likely to mediate at least some of the effects of stress on GnRH secretion in non-rodent species (Tilbrook et al., 2002). Therefore, it is reasonable to conclude that stress-induced reproductive dysfunction may be, at least in part, caused by a suppression of serotonergic projections to the GnRH neurons in the hypothalamus that normally support the pulsatile release of this neurohormone.

In this non-human primate model of stress-induced reproductive dysfunction, our laboratories have shown that serotonergic activity is suppressed in monkeys that are sensitive to reproductive dysfunction caused by exposure to mild psychosocial + metabolic stress. Stress-sensitive animals not only show a decrease in the physiological release of serotonin (Bethea et al., 2005a), but also decreases in the expression of several genes in the serotonin pathway. Bethea and colleagues (2005b) have reported that expression of many genes in the serotonin pathway are downregulated in the DRN of stress-sensitive individuals, including those related to synthesis (tryptophan hydroxylase-2; TPH2), reuptake (serotonin transporter; SERT), and degradation (monoamine oxidase A and B; MAO-A, MAO-B), and that there are fewer serotonin neurons in the dorsal raphe nucleus. Interestingly, while expression of the presynaptic autoreceptor $5HT_{1a}$ does not differ between monkeys with different sensitivity to stress-induced reproductive dysfunction (Bethea et al., 2005b), expression of the postsynaptic receptors $5HT_{2a}$ and $5HT_{2c}$ is elevated in the brains of stress-sensitive monkeys (Centeno et al., 2007), possibly as a compensation for suppressed circulating levels of serotonin in the brain. Recently, Bethea and colleagues have reported that stress-sensitive monkeys even show deficits in the expression of FEV-1, a "master gene" controlling serotonin neuron differentiation (Cameron et al., 2009). The most likely explanation for the widespread downregulation of these serotonin-related genes, even those with seemingly opposing functional effects (e.g., TPH2 for synthesis and MAO-A for degradation) is that stress-sensitive monkeys have fewer serotonergic neurons in the dorsal raphe nucleus as compared to highly stress-resilient monkeys. Therefore, it is possible that the decrease in serotonergic gene expression observed in SS monkeys is more of a function of increased

apoptosis of serotonin neurons rather than decreased functional activity of the neurons themselves. Nevertheless, Bethea and colleagues (2008) reported that even with accounting for the fewer number of serotonin-positive neurons in the dorsal raphe of SS monkeys, there is decreased expression of FEV1 per cell as compared to HSR monkeys. This suggests that the serotonin neurons that do survive in SS monkeys are still not as functional as those in the dorsal raphe of HSR animals.

The CRH system is an important neural network that regulates the innervation of serotonin neurons in the dorsal raphe nucleus (Cole & Sawchenko, 2002). Likewise, serotonin neurons originating in the raphe nuclei also project to the hypothalamic CRH system (Fuller, 1992) as one of many projections to corticolimbic sites involved in the stress response including the medial pre-frontal cortex (mPFC), septum, amygdala, and hippocampus (Ma et al., 1991; Steinbusch, 1981). Intracerebroventricular administration of CRH has been shown to inhibit serotonin release and raphe neuronal activity (Price et al., 1998). Interestingly, our laboratories have reported increased CRH gene expression in the caudal PVN (Centeno et al., 2007a) and CRH protein in the amygdala (Centeno et al., 2007b) of stress-sensitive individuals. These are two areas that have been shown to project to the brainstem in the rodent (Luiten et al., 1985; Puder & Papka, 2001; Sawchenko & Swanson, 1982), the anatomical location of the raphe nuclei. Because of the known pathways involving the amygdala and PVN, the raphe nuclei and the mediobasal hypothalamus, it is possible that GnRH neurons could be influenced by the CRH-induced suppression of serotonergic activity in stress-sensitive individuals (Dobson et al., 2003), although direct evidence for this is still lacking. My finding that monkeys sensitive to stress-induced reproductive dysfunction have increased CRH neuronal fiber

density in the dorsal and median raphe nuclei certainly lend support to this claim. It is possible that the increase in CRH-immunopositive fiber staining in the raphe nuclei of SS monkeys does not actually reflect increased release of CRH neurotransmitter to the raphe nuclei, but rather an increase in stored peptide within the neuronal fibers due to less release at the terminal. Taken together with previous reports of increased CRH gene expression and protein in the caudal PVN and amygdala (Centeno et al., 2007a,b), two areas which project to the raphe nuclei and inhibit serotonin activity, as well as our laboratory's finding that SS monkeys have decreased physiological release of serotonin (Bethea et al., 2005a) and less expression of serotonin-related genes (Bethea et al., 2005b), there is indirect evidence that this alternative explanation is not accurate in this case. However, further studies examining specific electrophysiological activity of serotonin neurons in the raphe nuclei of stress-sensitive and stress-resilient monkeys would be necessary to confirm these findings.

My findings in Chapter 2 that baseline HPA axis activity is not associated with stress-induced reproductive dysfunction and in Chapter 3 that the CRH-R1 antagonist antalarmin can partially improve acute reproductive axis function without preventing the stress-induced elevation in cortisol levels suggest that elevated central CRH activity in stress-sensitive monkeys is not suppressing reproductive axis activity via regulation of the HPA axis. Bethea and colleagues (2007a) had previously reported increased CRH gene expression in the PVN of stress-sensitive monkeys, yet in earlier chapters of this dissertation I found no evidence to support the hypothesis that elevated activity of the HPA axis was a primary neural mechanism underlying sensitivity to stress-induced reproductive dysfunction in female macaques. Therefore, the studies in this chapter

sought an explanation for the previously reported increases in CRH gene expression in SS monkeys. By far the most robust physiological and neurochemical distinction our laboratories have observed between stress-sensitive and more stress-resilient monkeys is in activity of the serotonin system. As such, this was an optimal candidate system to investigate for possible interaction with the CRH neurotransmitter system in these studies, as a possible explanation for upregulated CRH without increased HPA axis activation in stress-sensitive monkeys. It remains unknown whether this interaction is necessary to develop the phenotype of sensitivity to stress-induced reproductive dysfunction, or if alterations in the serotonin system are sufficient to produce this effect without contribution from CRH neuronal activation of the raphe nuclei. Nevertheless, these studies provide indirect evidence to support the hypothesis that CRH may be acting in a non-neuroendocrine manner to project to the raphe nuclei and suppress the serotonergic activation of GnRH secretion in the mediobasal hypothalamus.

Here I report differences in CRH-immunoreactive fibers in both the dorsal and the median raphe nucleus between SS and HSR monkeys, although previous studies involving suppression of serotonin-related genes in stress-sensitive monkeys were only reported in the dorsal raphe nucleus (Bethea et al., 2005a,b). While specific functional differences between the dorsal and median raphe are still largely unknown (Lechin et al., 2006), there is some indication that the dorsal raphe nucleus might be more directly involved in the regulation of the reproductive axis response to stress. The dorsal raphe nucleus in particular is extensively involved in the serotonergic innervation of most of the brain including the corticolimbic structures involved in the mediation of the stress response (Ma et al., 1991; Steinbusch, 1981), while median raphe serotonergic neurons

are more closely linked to innervation of subcortical structures (Lechin et al., 2006). Furthermore, the dorsal raphe has five times as many serotonin neurons as the median raphe does (Lechin et al., 2006), and the expression level of CRH receptors (both CRH-R1 and R2) is very high in the dorsal raphe (Mosko and Jacobs, 1975; Feldman and Conforti, 1980; Kant et al., 1983; Owens and Nemeroff, 1991; Hery et al., 2000; Lowry et al., 2000) especially when compared with the very low expression of CRH receptors in the median raphe (Le et al., 2002). Furthermore, while there is extensive interaction between levels of circulating ovarian steroid hormones and serotonin neural activity in the dorsal raphe nucleus, this does not seem to be the case for serotonergic activation in the median raphe (Bethea et al., 2002). This finding is particularly interesting as stress-sensitive monkeys have lower levels of both estrogen and progesterone (Bethea et al., 2008), and while low serotonin activity in these monkeys may be driving the suppression of ovarian hormones, it is also possible that low ovarian hormone levels in turn contribute to the low serotonin activity in these individuals. These results, together with my findings presented herein, support the hypothesis that increased CRH neuronal input to the raphe nuclei, and perhaps in particular to the dorsal raphe nucleus, may be acting to suppress GnRH neuronal activity in the hypothalamus by modulating serotonergic output from that region in stress-sensitive individuals.

Chapter 5

GENERAL DISCUSSION

5.1 Corticotropin-Releasing Hormone (CRH) Neurons Project to Numerous Extra-Hypothalamic Brain Areas to Regulate Stress-Related Behaviors and Physiological Processes

The studies in this dissertation addressed whether an increase in activity of the hypothalamic-pituitary-adrenal (HPA) axis constitutes an underlying neural mechanism that leads to the development of sensitivity to stress-induced reproductive dysfunction in female macaques. In Chapter 2 of this dissertation, I found that stress-sensitive and more stress-resilient monkeys did not differ in five of six measures of HPA axis activity: including basal diurnal secretion of adrenocorticotropic hormone (ACTH) and cortisol, ACTH and cortisol response to an acute intense psychological stressor, suppression of the HPA axis in response to glucocorticoid negative feedback, the integrative measures of cortisol concentration in hair, and adrenal weight. However, stress-sensitive monkeys did show greater activation of the HPA axis during the circadian light phase in response to mild psychosocial + metabolic stress, when compared to highly stress-resilient monkeys that maintain reproductive function when stressed. It is thus possible that the greater activation of the HPA axis in response to mild combined stress contributes in a causal manner to increased sensitivity of the reproductive axis to stress, although it is also possible there is simply a co-occurrence of greater stress-induced activation of the HPA axis and greater stress-induced suppression of the reproductive axis. Support for the latter hypothesis was provided in Chapter 3 where I reported that pharmacological blockade of

corticotropin-releasing hormone-1 receptors (CRH-R1) with antalarmin was able to prevent the stress-induced suppression of LH pulse frequency *without* suppressing the stress-induced rise in ACTH or cortisol secretion. This result indicated that activation of the HPA axis was *not* related to the acute stress-induced suppression of the reproductive axis. Following this up, the studies in Chapter 4 indicated that there is further, if indirect, evidence to suggest that CRH neurons may be acting in a non-neuroendocrine manner, through interaction with the serotonin system, to contribute to the stress-induced suppression of reproductive function in stress-sensitive monkeys through this mechanism.

The CRH system is a complex network of neurons that, in addition to having neuroendocrine action at the median eminence to regulate the HPA axis, project to numerous sites in the brain to regulate various stress-associated behaviors and physiological processes (Koob, 1999). In the paraventricular nucleus (PVN) of the hypothalamus alone, a region which is thought to contain the majority of neuroendocrine CRH neurons that regulate activation of the HPA axis (Koob, 1999), there is regional organization by which a subset of CRH neurons in the PVN do not primarily function in this capacity (Swanson & Kuypers, 1980; Kiss et al., 1983). Non-neuroendocrine CRH neurons in the PVN project to a variety of limbic and autonomic areas outside the median eminence, including the bed nucleus of the stria terminalis (BNST) and central amygdala (Walker et al., 2001; Crane et al., 2003), the locus coeruleus (Chowdhury et al., 2000; Makino et al., 2002; Reyes et al., 2005), and the medulla and spinal cord (Sawchenko & Swanson, 1982; Luiten et al., 1985; Puder & Papka, 2001). CRH-immunoreactive neuronal bodies and fibers are in widespread distribution in extra-hypothalamic brain regions that are not directly involved with traditional neuroendocrine responses to

stressful stimuli (Swanson et al., 1983). Furthermore, the distribution of the two known CRH receptors, CRH-R1 and CRH-R2, has been mapped to various brain regions unrelated to pituitary activation (De Souza, 1987; Potter et al., 1994; Chalmers et al., 1995; Steckler & Holsboer, 1999). CRH-R1 and CRH-R2 receptors are thought to have different functional roles in the stress response (Steckler & Holsboer, 1999; Coste et al., 2001), and they exhibit different patterns of expression throughout the brain (Chalmers et al., 1995; Steckler & Holsboer, 1999). As a neurotransmitter, CRH is capable of activating (via CRH-R1 receptors) and maintaining or initiating recovery from (via CRH-R2 receptors) the stress response (Coste et al., 2001), the final behavioral or physiological consequences of which are highly dependent upon the localization of the receptor subtypes (Steckler & Holsboer, 1999). In the brain, CRH has been implicated in a multitude of processes, from increasing stress-related behaviors by activation of the amygdala and cortex, to adaptation of blood pressure, heart rate and motor function by activity in the brainstem, to suppressing immune and reproductive function by modulating neuronal activity in various subcortical regions (Dunn & Berridge, 1990; De Souza, 1995; Steckler & Holsboer, 1999). Production of CRH has also been reported in several peripheral locations, including placenta, heart and various muscle tissue, immune cells, and blood vessels (Chrousos et al., 1998; Steckler & Holsboer, 1999; Kalantaridou et al., 2007), although the specific function and mechanism of action of CRH in each of these tissues is still under investigation.

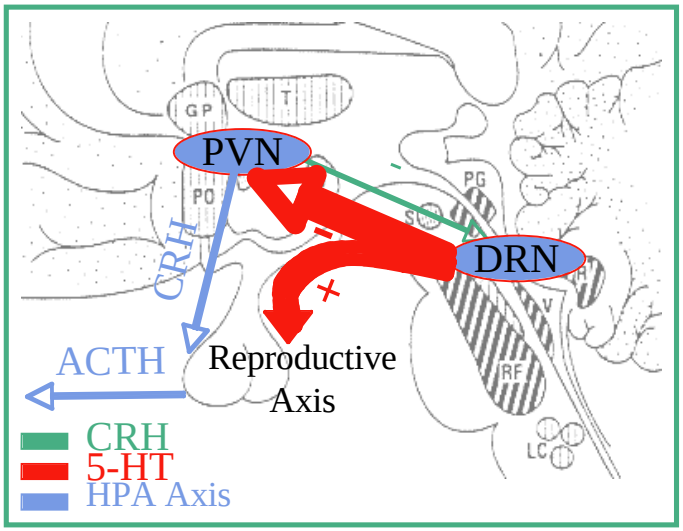
In addition to its direct role in mediating stress-related processes in the brain, including stress-induced reproductive dysfunction, CRH is thought to interact with other neurotransmitter systems to integrate various autonomic and behavioral components of

the stress response (Dunn & Berridge, 1990; Valentino et al., 1993). CRH neuronal projections from various nuclei have bidirectional connectivity with most of the limbic and autonomic brain areas, many of which have been implicated in the regulation of the reproductive axis (Swanson, 1987; Dobson et al., 2003). As such, elevated CRH neuronal activity *per se* does not necessarily indicate increased activation of the HPA axis, nor does it preclude a role for integration of other signaling systems in the development of sensitivity to stress-induced reproductive dysfunction.

While activation of the adrenal glands has received a good deal of attention regarding its role in regulating stress responses, the HPA axis is not the sole mediator of the physiological response to stressful stimuli, nor is it a system which acts in isolation. Many signaling systems play an integrative role in regulating stress responses, including the urocortin, arginine/vasopressin, opioid, GABA, noradrenaline, dopamine and serotonin systems (McEwen, 2008). In addition, each of these neural mechanisms may have a role in regulating activity of the reproductive axis, both through direct pathways affecting GnRH neuronal activity, as well as through interactions with the CRH system (Dobson et al., 2003). For example, CRH administered either intracerebroventricularly (i.c.v.) or directly into the locus coeruleus (LC) increases the electrophysiological firing rate of neurons in this region, and the release of noradrenaline in a variety of brain regions to which the LC projects (Koob, 1999). As both adrenaline and noradrenaline are known to suppress LH pulse frequency and amplitude in rats and sheep (Goodman et al., 1996; Smith & Jennes, 2001), this suggests a possible role for CRH-adrenaline/noradrenaline in the mediation of stress-induced reproductive dysfunction. Interestingly, coadministration of naloxone, a μ-opioid receptor antagonist, into the

hypothalamus can block the CRH-induced suppression of GnRH secretion into portal blood in both rats and monkeys (Rivest & Rivier, 1991; Phogat et al., 1997), indicating a possible antagonistic relationship between the CRH and opioid signaling systems in the effects of stress on reproduction. Furthermore, GABA receptors are present on 95% of CRH neurons (Cullinan, 2000), and GABA administered directly into the PVN increases CRH production in microdialysate from that region (Cole & Sawchenko, 2002), indicating both a direct and an indirect role for GABA-mediated GnRH neuronal activity, as GABAergic neurons also make direct synaptic connectivity to GNRH neurons in the arcuate nucleus (Dobson et al., 2003). Together with previous reports from our laboratory indicating differential activity of the serotonin system in the brains of stress-sensitive macaques (Bethea et al., 2005a,b), the results of the studies presented in this dissertation suggest a possible interactive role of the serotonin and CRH systems in the development of sensitivity to stress-induced reproductive dysfunction in female monkeys. If such an interaction of the CRH and serotonin systems is indeed causal in the sensitivity of the reproductive axis to stress in women and female monkeys, it proposes a new working model for a potential neural mechanism underlying the etiology of stress-induced amenorrhea (Figure 5.1). This mechanism would indicate that the reciprocal innervation of the paraventricular nucleus of the hypothalamus (PVN) and dorsal raphe nucleus (DRN) by CRH and serotonin, respectively, is different in stress-sensitive vs. stress-resilient individuals. Specifically, stress-sensitive individuals have increased inhibitory CRH neuronal input to the DRN, resulting in decreased serotonin release and less stimulation of the reproductive axis. In contrast, stress-resilient individuals have

(A)



(B)

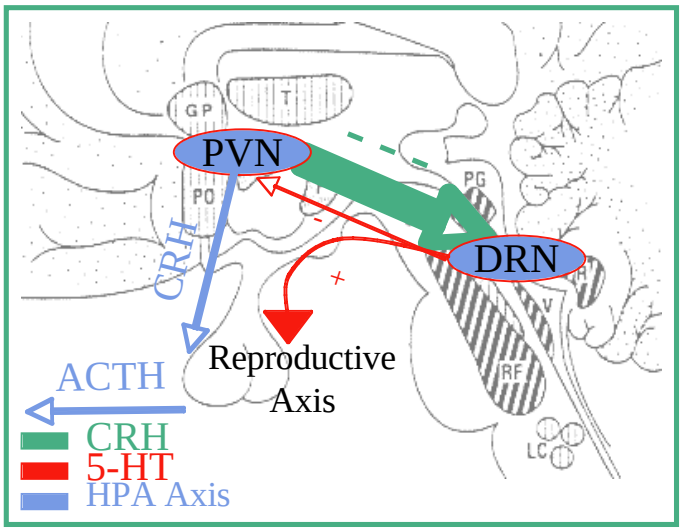


Figure 5.1 Working model of potential neural mechanism underlying individual differences in sensitivity to stress-induced reproductive dysfunction in (A) stress-resilient and (B) stress-sensitive individuals.

decreased inhibitory CRH neuronal input to the DRN, resulting in less inhibition of that nucleus, and increased stimulation of the reproductive axis by serotonin.

Other studies have suggested a role for serotonin and CRH interactions in the development of stress-sensitivity, as polymorphism variations in the serotonin transporter (SERT) and monoamine oxidase A (MAO-A) genes have been associated with differences in the behavioral and HPA responses to stress, as well as in susceptibility to major depression (Jabbi et al., 2007). In this study, Jabbi and colleagues report that individuals carrying the short/short allelic variation in the promoter region of the SERT gene, which has been associated with various traits of anxious behavior (Lesch et al., 1996; Courtet et al., 2001), and the low-functioning allele for the MAO-A gene showed the largest increase in both ACTH and cortisol response to a psychological stressor. Furthermore, Nappi and colleagues (1993) reported suppressed physiological responsivity to naloxone and fenfluramine challenge as compared to healthy controls and proposed that a central disruption of opioidergic and serotonergic mediation of the HPA axis may be a possible neural mechanism underlying the etiology of stress-induced amenorrhea, and may explain the increased secretion of cortisol observed in some studies of women with this disorder (Biller et al., 1990; Berga et al., 1997; Meczekalski et al., 2000; Kondoh et al., 2001; Lindahl et al., 2007). While there are certainly multiple neurotransmitter systems and regulatory factors which warrant further investigation as an underlying causal mechanism for the development of stress-induced amenorrhea, the findings of a serotonin-CRH interaction presented herein may be promising in terms of developing future treatment options for patients with this disorder.

5.2 Future Directions for Studying the Neurotransmitter Versus Neuroendocrine Hormone Roles of CRH in Regulating Stress-Induced Reproductive Dysfunction

As discussed previously, the CRH system is an extensive neuronal network that can act both as a neuroendocrine hormone to regulate the HPA axis, as well as a neurotransmitter to project to numerous sites in the brain to mediate various stress responses (Koob, 1999). The studies in this dissertation provide physiological, pharmacological, and immunohistochemical data that suggests that the HPA axis, as regulated by CRH acting as a neurohormone, does not provide the causal mechanism underlying sensitivity to stress-induced reproductive dysfunction. However, CRH acting as a neurotransmitter may be interacting with the serotonin system to contribute to the development of stress-sensitivity in female macaques. One way of further exploring such a role of CRH to increase the sensitivity of the reproductive axis to stress via modulation of serotonin neurons would be to expand on the pharmacological studies in Chapter 3 using the specific CRH-R1 receptor antagonist, antalarmin. In Chapter 3 I utilized systemic administration of antalarmin to assess the differences in both HPA and reproductive axis activity following exposure to acute mild combined stress in stress-sensitive (SS), medium stress-resilient (MSR), and highly stress-resilient (HSR) monkeys. As I observed a prevention of stress-induced suppression of LH pulse frequency without a concomitant suppression of stress-induced secretion of cortisol, the data suggests that a) ACTH and cortisol, as part of the HPA axis, do not play a causal role in the acute suppression of LH pulse frequency observed in stress-sensitive monkeys, and b) antalarmin at the dose administered (10 mg/kg i.v.) may have been acting as a partial agonist or incomplete antagonist at the CRH-R1 receptors in the anterior pituitary,

as treatment with antalarmin was not able to prevent the stress-induced secretion of ACTH or cortisol. However, in order to further support my hypothesis that non-neuroendocrine CRH neurons are interacting with the serotonin system to affect LH pulse frequency, it would be important to replicate the findings of this study using local administration of antalarmin directly into the raphe nuclei (possibly using headcap-mounted guide cannulaes for microinjection). If the findings I report in Chapter 3 were replicated, it would lend further support to the conclusions drawn in this dissertation. Additionally, it would be possible to conduct a similar study in which antalarmin was locally administered to the pituitary using a similar technique in animals with cytotoxic or electrolytic lesions of the dorsal and median raphe nuclei. In such a study, ablation of the raphe nuclei would prohibit any signal transduction pathway from non-neuroendocrine CRH neurons in the PVN to serotonin neurons in the DRN/MRN to the GnRH neurons in the arcuate, thereby ruling out this pathway as a means to suppress LH pulse frequency following exposure to stress. Although this would not specifically isolate CRH-induced stimulation of the HPA axis, as CRH neuronal projections to regions other than the dorsal and median raphe (such as the locus coeruleus and BNST) would still be possible, it would rule out the CRH-serotonin-GnRH pathway isolated in the first experiment proposed. As such, direct comparison of the results of these two experiments would more specifically indicate a CRH signaling pathway that might be contributing to the sensitivity to stress-induced reproductive dysfunction. To explore the contribution of the serotonin system to the development of stress-sensitivity in a broader sense, one could also treat either locally or systemically with a serotonin enhancing agent such as an SSRI (specific serotonin reuptake inhibitor) in stress-sensitive and more stress-

resilient female monkeys to ask whether reversing the low serotonin our laboratory has observed in stress-sensitive monkeys would improve resiliency of the reproductive axis to stress. This method has the added benefit of also being able to conduct identical studies in women, as this class of drugs has Food and Drug Administration approval for use in humans. In fact, these experiments have already been proposed for future study in our laboratories in collaboration with clinical investigators. If this line of reasoning is correct, and the serotonin system is heavily implicated as an underlying neural mechanism in the development of sensitivity to stress-induced reproductive dysfunction, it could greatly advance the treatment options for women with stress-induced amenorrhea and improve the clinical outcome for infertility cases worldwide.

5.3 Other Factors Influencing Sensitivity to Stress-Induced Reproductive Dysfunction

Many factors can influence individual differences in stress-sensitivity that have not been addressed by the studies in this dissertation, but are nevertheless important to the full understanding of the etiology of stress-induced reproductive dysfunction. Gene by environment by development interactions are likely to be pivotal to understand how a particular individual will function not only in a healthy normal state, but also in a variety of disease states. In addition to numerous neural mechanisms that underlie individual differences in the sensitivity to stress-induced reproductive dysfunction, genetics, early life stress, ovarian steroid hormonal milieu, behavioral temperament and behavioral dominance are important variables that are likely to impact the pathophysiology of stress-

induced reproductive dysfunction, in clinical settings as well as in experimental animal models.

Despite a recent accumulation of data indicating a major contribution of genetics to the development of psychopathology, efforts to directly match psychopathological states to specific genotypes has remained largely unsuccessful (Moffitt et al., 2005; Caspi & Moffitt, 2006; Meyer-Lindenberg & Weinberger, 2006). While there is some indication of a familial contribution to more organic forms of GnRH deficiency, such as Kallmann's syndrome (Kottler et al., 2004), the role of genetics in the etiology of stress-induced amenorrhea remains largely unknown (Warren & Fried, 2001). However, work on heritability and genetic contribution to specific endophenotypes of stress-induced amenorrhea have provided some insight into the possible involvement of several serotonin-related genes. Polymorphism variation of the promoter region of the SERT gene, the mRNA expression of which is downregulated in stress-sensitive monkeys (Bethea et al., 2005a), has been reported to be somewhat heritable, with greater similarity of 5HTTPR polymorphism observed in siblings as compared to non-related individuals (Mazzanti et al., 1998). The short allele of the 5HTTPR polymorphism has lower transcriptional activity, leading to reduced mRNA levels (Collier et al., 1996), and functionally has been linked to anxiety-related behaviors in both humans (Lesch et al., 1996) and monkeys (Bethea et al., 2004). Additionally, the heritability for anorexia nervosa has been estimated at 0.7 (Ben-Dor et al., 2002), indicating a strong familial component to the development of this eating disorder, which is prevalent subclinically in the population of women with stress-induced amenorrhea (Warren et al., 1999).

Much like the role of genetic contribution, exposure to early life stress can be influential in the development of neural pathways and behavioral and physiological responses to stress (Kaufman et al., 2000; Levine S, 2000; Cicchetti & Rogosch, 2002). It has been suggested that early life stress increases vulnerability to psychopathological states such as major depression and anxiety, with the sensitivity to these disorders differing depending on genetic and environmental variables (Charney, 2004; Plotsky et al., 1998). Fortunately, much more is known about the prevalence of early life stress as compared to heritability in the population of women with stress-induced amenorrhea, although these findings are by self-report and do not provide evidence as to causality. Adverse childhood experiences are commonly reported in clinical populations of adult women with stress-induced amenorrhea, ranging from increased social stress to sexual molestation (Liu, 1990). Interestingly, various forms of early life stress have also been reported to precede the onset of stress-induced amenorrhea in adolescent girls (Bomba et al., 2007). In this study, in which parental interviews were conducted to verify the presence of stressful early life events, 90% of the adolescent patients' parents reported an incidence of psychopathology in at least one immediate family member in their home, 80% reported postpartum depression, 55% described significant family conflict including domestic violence, and 50% of these parents were suffering from chronic disease during the patient's first years of life. Additionally, Facchinetti and colleagues (1993) have reported that in nearly half of interviewed patients with stress-induced amenorrhea, onset of reproductive dysfunction was associated with a significant psychosocially stressful life event during adolescence, a period in which the developing brain is particularly sensitive to environmental stressors (Dahl & Gunnar, 2009).

Animal models attempting to characterize the effects of early life stress have begun to elucidate the neurobiological underpinnings of the consequences of early life stress, again focusing on endophenotypes of various disorders. Maternal separation in rhesus macaques results in increased anxiety and abnormal social behavior, changes in neuroanatomy of the prefrontal cortex and altered gene expression in the amygdala, and the timing of this early life stress is important to the development of different consequences (Cameron, 2004; Sabatini et al., 2007). Other models of early deprivation and social stress in non-human primates have identified neurochemical consequence in disrupted neurotransmitter signaling including serotonin, CRH, norepinephrine, and dopamine systems (Higley et al., 1992; Higley et al., 1996), as well as in transmission of the neuropeptides oxytocin and vasopressin, important for mediating social bonding and stress-reactivity (Winslow, 2005). Exposure to early life stress appears to contribute to the pathophysiology of stress-induced amenorrhea, and future animal models may further characterize a mechanism for this phenomenon through experimental manipulation that is not feasible in associative clinical studies.

Just as it has been well documented that stress affects production of ovarian steroid hormones via inhibition of the GnRH pulse generator (Vigersky et al., 1997; Loucks et al., 1989; Bronson & Manning, 1991; Wade & Schneider, 1992; Foster et al., 1989; Berga & Girton, 1989; Reame et al., 1985; Rasmussen et al., 1986; Miller et al., 1993), recent evidence has suggested that in turn, ovarian steroid hormones can affect the sensitivity of and the physiological responses of an individual to stressful stimuli (Bethea et al., 1999). While accumulating research will undoubtedly implicate numerous neurotransmitter and neuropeptide systems, three of the best studied neural mechanisms

that interact with ovarian hormones to affect individual differences in the allostatic load of external stressors are the CRH, beta-endorphin and serotonin signaling systems.

Kirschbaum and colleagues (1999) have suggested that the activity of the HPA axis is heavily influenced by the particular hormonal milieu of an individual. Glucocorticoid response to both psychosocial and physical (cold press) stressors was enhanced in women during the luteal phase of their menstrual cycle, when ovarian steroid hormones are abundant, dominated by the release of progesterone from the corpus luteum (Tersman et al., 1991). Estrogen response elements (EREs) have been shown to colocalize with the CRH promoter, suggesting that estrogen availability may mediate CRH production and/or release (Haeger et al., 2006). In addition, exogenous estrogen has been shown to further stimulate CRH synthesis in the PVN of both rats (Patchev et al., 1995) and monkeys (Roy et al., 1999).

Beta-endorphin has also been shown to inhibit GnRH neuronal activity, particularly during the luteal phase of the menstrual cycle when circulating levels of progesterone are high (Ferin & Vande Wiele, 1984). It has been suggested that beta-endorphin is the negative feedback mechanism by which ovarian steroid hormones are able to suppress GnRH firing and subsequent LH pulse frequency (Taylor et al., 2007). Naloxone, a μ-opioid receptor antagonist, has been shown to reverse stress-induced suppression of LH secretion in adult, but not prepubertal monkeys (Blank & Murphy, 1991), and the degree of LH restoration by naloxone was directly correlated with circulating steroid hormone levels, suggesting that the presence of ovarian steroid hormones may also aid in reversing the effects of beta-endorphin on the reproductive axis. One possible explanation for the seemingly contradictory effects of steroid

hormone mediation of beta-endorphin on the HPG axis is that beta-endorphin is both a neurotransmitter and a pituitary hormone (Meczekalski et al., 2008). While there is ample evidence to suggest that hypothalamic beta-endorphin suppresses GnRH neuronal activity, there is no indication that beta-endorphin hormone released from the pituitary has a similar effect (Dudas & Merchenthaler, 2004).

Lastly, ovarian steroid hormones may also influence individual differences in stress sensitivity because of their involvement in regulating the serotonin system in rodents and non-human primates (Alves et al., 1998; Bethea et al., 1999). Estrogen treatment in ovariectomized rodents show significant upregulation of the 5-HT_{1b} receptor and the serotonin transporter, SERT, as compared with ovariectomy alone (Fink et al., 1998). Progesterone treatment in estrogen primed, ovariectomized monkeys increases fenfluramine-induced serotonin microdialysate from the mediobasal hypothalamus (Centeno et al., 2007), where serotonin might mediate the neuronal activity of GnRH neurons. Estrogen and progesterone also mediates expression of 5-HT_{1a} and 5-HT_{2c} receptors in the dorsal raphe (Henderson and Bethea, 2008). Treatment with estrogen also increases gene expression (Pecins-Thompson et al., 1996) and protein (Bethea et al., 2000) of tryptophan hydroxylase (TPH), the rate-limiting enzyme for serotonin production, in the dorsal raphe nucleus. Finally, in the same animal model, estrogen and progesterone treatment reduces expression of the serotonin transporter (SERT) mRNA (Pecins-Thompson et al., 1998), which is involved in clearing serotonin from the synapse. As is the case with all the mechanisms discussed here, the interaction between ovarian steroid hormones and various neurotransmitter systems is bidirectional, therefore it is extremely difficult to determine causality in characterizing the neural mechanism for

stress-sensitivity and reproductive dysfunction. Further research is needed to parse out the effects of hormonal milieu on stress-sensitivity as mediated through these proposed systems, as it is likely that hormones exert different effects through different mechanisms, including those not discussed here, and depend greatly on both the nature and intensity of the stressor.

Behavioral temperament is yet another factor which may affect the pathophysiology of stress-induced reproductive dysfunction (Liu & Bill, 2008). Depressive and anxious temperaments have been reported in women with stress-induced amenorrhea (Bomba et al., 2007; Marcus et al., 2001), although these symptoms range from subclinical to psychopathological and as such the specific contribution of temperament to stress-sensitivity in this population remains unknown. It has been suggested that certain behavioral traits such as fearful and anxious temperaments are associated with increased stress-reactivity (Tyrka et al., 2008; Gunnar et al., 2009), and that this relationship is evident in as early as pre-school aged children (Talge et al., 2008). Experimental animal models can help to further characterize this relationship between temperament and sensitivity to stress by associating these findings with genetic and neurochemical alterations. A socially submissive temperament and conflict avoidance has also been associated with sensitivity to stress in rats (Steimer et al., 1997), as well as in monkeys (Shively et al., 1997a). Interestingly, in this non-human primate model, submissive temperament and stress-reactivity have also been related to differences in dopaminergic activity (Shively et al., 1997b), serotonin synthesis and reuptake (Shively et al., 2003), increased heart rate, “depressive” behavior and low levels of ovarian steroid hormones (Shively et al., 2005). However, a meta-analysis investigating dominance rank

and stress physiology among seven different species of non-human primates reported that more subordinate monkeys do not always exhibit greater stress reactivity (as measured by increased cortisol levels to stressful stimuli) than more dominant monkeys, and that this is highly dependent upon the species studied (Abbott et al., 2003). Though there is a good deal of evidence suggesting a relationship between temperament and stress-sensitivity, it remains unclear whether either factor is causal in the etiology of stress-induced reproductive dysfunction, and further research is needed to clarify the role of temperament in this and other disorders. While this dissertation work does not assess the contribution of genetics, early life stress, ovarian steroid hormonal milieu or temperament to the development of sensitivity to stress-induced reproductive dysfunction in this animal model, any conclusions regarding neural mechanisms of this disorder must be interpreted bearing these factors, and others, in mind.

5.4 Summary and Conclusions

My dissertation studies have contributed several important findings that further characterize stress-sensitive individuals, showing that stress-sensitive animals have lower LH pulse frequency indicating dysfunction of the HPG axis at the level of the pituitary or higher, and increased acute cortisol response to our mild combined stress paradigm during the circadian light phase. It was the overall goal of my dissertation to test the hypothesis that the HPA axis plays a critical role in mediating the effects of stress on the reproductive axis, particularly in stress-sensitive individuals. However, the results of the pharmacological studies in Chapter 3 suggest that the HPA axis is not mediating stress-

induced suppression of the reproductive axis. Alternatively, both Chapter 3 and Chapter 4 provide evidence for a non-neuroendocrine role of CRH in mediating stress-induced reproductive dysfunction in the most stress-sensitive monkeys. Specifically, CRH-induced activation of the HPA axis to elevate ACTH and cortisol does *not* seem to be the primary mechanism by which individuals may develop sensitivity to stress-induced reproductive dysfunction. However, my results suggest that CRH appears to be acting as a neurotransmitter to modulate the physiological activity of serotonin and possibly other neurotransmitter systems, which may be influencing the reproductive sensitivity to stress in the primate.

This dissertation adds to a body of previous work in this non-human primate model of stress-induced reproductive dysfunction that has already and will likely continue to make important clinical contributions to the treatment of stress-induced amenorrhea. Early treatment strategies for reversing the effects of stress-induced amenorrhea involved the highly invasive and technically complex pulsatile infusion of GnRH, with limited success in conception and pregnancy (Guoth & Zsolnai, 1986; Couzinet et al., 1986). As the field began to look further into the etiology of stress-induced amenorrhea, initial research suggested the disorder was caused by psychological and/or behavioral responses to stressful life events leading to a suppression of GnRH neuronal activity (Suh et al., 1988; Monzani et al., 1989; Biller et al., 1990). Based on these studies, clinicians have explored the non-invasive treatment strategy of cognitive behavioral therapy (Berga, 1997), again with limited success. However, mounting evidence later indicated that women with stress-induced amenorrhea experienced a suppression of reproductive function in response to psychosocial *and* metabolic stress

(Berga et al., 1997; Laughlin et al., 1998; Warren et al., 1999; Warren & Fried, 2001; Perkins et al., 2001). Experimental work in our non-human primate model of stress-induced amenorrhea further elucidated the etiology of this disorder with reports that the combination of mild psychosocial plus concomitant diet and exercise, as part of the clinical profile of women with stress-induced amenorrhea, is synergistic to suppress reproductive function (Williams et al., 2007), especially in a subset of stress-sensitive individuals (Cameron, 1997; Bethea et al., 2005a; Bethea et al., 2005b; Centeno et al., 2007a; Centeno et al., 2007b; Bethea et al., 2008). Furthermore, work in our lab has shown that the effects of exercise-induced amenorrhea can be reversed by increasing available caloric intake (Williams et al., 2001). As such, new treatment strategies have begun to simultaneously implement cognitive behavioral therapy focused on addressing coping strategies for psychosocial stressors and attitudes towards food and body image with a change in diet that increases daily caloric intake, and the outcome of this recent change in treatment strategy looks very promising (Berga et al., 2003; Berga & Loucks, 2006).

While this approach targeting both psychosocial and metabolic stressors has been successful in improving fertility in women with stress-induced amenorrhea, it does not address the neural mechanisms thought to underlie the development of this disorder. Recent collaborative work in this model has suggested that a downregulation of serotonergic neural networks may be, at least in part, causing a suppression of GnRH neuronal activity in stress-sensitive monkeys (Bethea et al., 2005a; Bethea et al., 2005b; Cameron et al., 2009). Furthermore, this dissertation work indicates that interaction between the CRH system and serotonergic networks may be an important relationship

that causes psychosocial and metabolic stresses to impinge upon reproductive function. Based on these findings, future work in the Cameron lab, in collaboration with clinical colleague Sarah Berga, MD, will investigate the ability of citalopram, a specific serotonin reuptake inhibitor (SSRI), to improve fertility rates in both stress-sensitive monkeys and in women with stress-induced amenorrhea. Preliminary findings in our lab have shown that citalopram treatment increased peak levels of estrogen in the follicular phase and progesterone in the luteal phase in stress-sensitive monkeys (Cameron et al., 2004), providing sound rationale for the further investigation into the possible reproductive benefits of this FDA-approved drug. If these studies are successful in improving fertility in monkeys and in women with stress-induced amenorrhea, it would greatly benefit the clinical field, providing an effective, inexpensive alternative to in vitro fertilization or other more costly and invasive strategies for treating infertility in these women.

In conclusion, I find that activation of the HPA axis is not the primary neural mechanism underlying sensitivity to stress-induced reproductive dysfunction in female cynomolgus monkeys. Furthermore, this dissertation work provides evidence that CRH neurons may be acting in a non-neuroendocrine manner to project to the raphe nuclei to suppress serotonin output and further impinge upon the reproductive axis in stress-sensitive monkeys. This work, in addition to previous studies in this monkey model of stress-induced amenorrhea, has direct and important clinical relevance and will continue to substantially contribute to the treatment of stress-induced reproductive dysfunction and resulting infertility in women.

Chapter 6

REFERENCES

Abbott DH, Keverne EB, Bercovitch FB, Shively CA, Mendoza SP, Saltzman W, Snowdon CT, Ziegler TE, Banjevic M, Garland Jr. T and Sapolsky RM (2003). Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Horm Behav **43**(1): 67-82.

Afione S, Duvilanski B, Seilicovich A, Lasaga M, Diaz MC and Debeljuk L (1990). Effects of serotonin on the hypothalamic-pituitary GABAergic system. Brain Res Bull **25**(2): 245-9.

Alderfer BS and Allen MH (2003). Treatment of agitation in bipolar disorder across the life cycle. J Clin Psychiatry **64**(Suppl 4): 3-9.

Alves SE, Weiland NG, Hayashi S and McEwen B (1998). Immunocytochemical localization of nuclear estrogen receptors and progestin receptors within the dorsal raphe nucleus. J Comp Neurol **391**: 322-34.

American Psychiatric Association (2000). Diagnostic and Statistical Manual of Mental Disorders, 4th ed. Revised. American Psychiatric Press: Washington, DC.

Amin HK and Hunter WM (1970). Human pituitary follicle-stimulating hormone: distribuion, plasma clearance and urinary excretion as determined by radioimmunoassay. J Endocrinol **48**(3): 307-17.

Antoni FA, Palkovits M, Makara GB, Linton EA, Lowry PJ and Kiss JZ (1983). Immunoreactive corticotropin-releasing hormone in the hypothalamoinfundibular tract. Neuroendocrinol **36**(6): 415-23.

Arimura A, Kastin AJ, Gonzalez-Barcena D, Siller J Weaver RE and Schally AV (1974). Disappearance of LH-releasing hormone in man as determined by radioimmunoassay. Clin Endocrinol **3**:421-5.

Ayala AR, Pushkas J, Higley JD, Ronsaville D, Gold PW, Chrousos GP, Pacak K, Calis KA, Gerald M, Lindell S, Rice KC and Cizza G (2004). Behavioral, adrenal, and sympathetic responses to long-term administration of an oral corticotropin-releasing hormone receptor antagonist in a primate stress paradigm. J Clin Endocrinol Metab **89**(11): 5729-37.

Baldwin DM (1979). The effect of glucocorticoids on estrogen-dependent luteinizing hormone release in the ovariectoized rat and on gonadotropin secretion in the intact female rat. Endocrinol **105**: 120-8.

Baldwin DM and Sawyer CH (1974). Effects of dexamethasone on LH release and ovulation in the cyclic rat. Endocrinol **94**: 1397-1403.

Baldwin DV and Suomi SJ (1974). Reactions of infant monkeys to social and nonsocial stimuli. Folia Primatol (Basel) **22**(4): 307-14.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW and Lee KF (2000). Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behavior and are hypersensitive to stress. Nat Genet **24**(4): 410-4.

Barrell GK (2007). Immunological influences on reproductive neuroendocrinology. Soc Reprod Fertil Suppl. **64**: 109-22.

Barron JL, Millar RP and Searle D (1982). Metabolic clearance and plasma half-disappearance time of D-TRP6 and exogenous luteinizing hormone-releasing hormone. J Clin Endocrinol Metab **54**(6): 1169-73.

Barroso G, Oehninger S, Monzo A, Kolm P, Gibbons WE and Muasher SJ (2001). High FSH:LH ratio and low LH levels in asal cycle day 3: impact on follicular development and IVF outcome. J Assist Reprod Genet **18**:499-505.

Bassett JL and Foote SL (1992). Distribution of corticotropin-releasing factor-like immunoreactivity in squirrel monkey (Saimiri sciureus) amygdala. J Comp Neurol **323**: 91-102.

Beck AT, Ward CH, Mendelson M, Mock J and Erbaugh J (1961). An inventory for measuring depression. Arch Gen Psychiatry **4**: 561-71.

Belchetz PE, Plant TM, Nakai Y, Keogh EJ and Knobil E (1978). Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. Science **202**(4368): 631-3.

Ben-Dor DH, Laufer N, Apter A, Frisch A and Weizman A (2002). Heritability, genetics and association findings in anorexia nervosa. Isr J Psychiatry Relat Sci **39**(4): 262-70.

Berga SL (1997). Behaviorally induced reproductive compromise in women and men. Semin Reprod Endocrinol **15**(1): 47-53.

Berga SL, Daniels TL and Giles DE (1997). Women with functional hypothalamic amenorrhea but not other forms of anovulation display amplified cortisol concentrations. Fertil Steril **67**: 1024-30.

Berga SL and Girton LG (1989). The psychoneuroendocrinology of functional hypothalamic amenorrhea. Psychiatr Clin North Am **12**: 105-16.

Berga SL and Loucks TL (2006). Use of cognitive behavior therapy for functional hypothalamic amenorrhea. Ann N Y Acad Sci **1092**: 114-29.

Berga SL, Loucks-Daniels TL, Adler LJ, Chrousos GP, Cameron JL, Matthews KA, and Marcus MD (2000). Cerebrospinal fluid levels of corticotrophin-releasing hormone in women with functional hypothalamic amenorrhea. Am J Obstet Gynecol **182**(4): 776-84.

Berga SL, Marcus MD, Loucks TL, Hlastala S, Ringham R and Krohn MA (2003). Recovery of ovarian activity in women with functional hypothalamic amenorrhea who were treated with cognitive behavior therapy. Fertil Steril **80**: 976-981.

Berga SL, Mortola JF, Girton L, Suh B, Laughlin G, Pham P and Yen SSC (1989). Neuroendocrine aberrations in women with functional hypothalamic amenorrhea. J Clin Endocrinol Metab **68**(2): 301-8.

Bethea CL and Centeno ML (2008). Ovarian steroid treatment decreases corticotropin-releasing hormone (CRH) mRNA and protein in the hypothalamic paraventricular nucleus of ovariectomized monkeys. Neuropsychopharmacol **33**(3): 546-56.

Bethea CL, Centeno ML and Cameron JL (2008). Neurobiology of stress-induced reproductive dysfunction in female macaques. Mol Neurobiol **38**: 199-230.

Bethea CL, Gundlah C and Mirkes SJ (2000). Ovarian steroid action in the serotonin neural system of macaques. Novartis Found Symp **230**: 112-30.

Bethea CL, Mirkes SJ, Shively CA and Adams MR (2000). Steroid regulation of tryptophan hydroxylase protein in the dorsal raphe of macaques. Biol Psychiatry **47**(6): 562-76.

Bethea CL, Pau FK, Fox S, Hess DL, Berga SL and Cameron JL (2005a). Sensitivity to stress-induced reproductive dysfunction linked to activity of the serotonin system. Fertil Steril **83**(1): 148-55.

Bethea CL, Pecins-Thompson M, Schutzer WE and Gundlah C (1999). Ovarian steroids and serotonin neural function. Mol Neurobiol **18**: 87-123.

Bethea CL, Streicher JM, Mirkes S, Sanchez RL, Reddy AP and Cameron JL (2005b). Serotonin-related gene expression in female monkeys with individual sensitivity to stress. Neuroscience **132**(1): 151-66.

Bethea CL, Streicher JM, Coleman K, Pau FK, Moessner R and Cameron JL (2004). Anxious behavior and fenfluramine-induced prolactin secretion in young rhesus macaques with different alleles of the serotonin reuptake transporter polymorphism (5HTTLPR). Behav Genet **34**(3): 295-307.

Biederman J, Rosenbaum JF, Hirshfeld DR, Faraone SV, Bolduc EA, Gersten M, Meminger SR, Kagan J, Snidman N and Reznick JS (1990). Psychiatric correlates of behavioral inhibition in young children of parents with and without psychiatric disorders. Arch Gen Psychiatry **47**(1): 21-6.

Biller BMK, Federoff HJ, Koenig JI and Klibanski A (1990). Abnormal cortisol secretion and responses to corticotropin-releasing hormone in women with hypothalamic amenorrhea. J Clin Endocrinol Metab **70**: 311-317.

Blank MS and Murphy JR (1991). Luteinizing hormone sensitivity to naloxone in maturing male chimpanzees. Brain Res Bull **27**: 241-5.

Boccuzzi G, Angeli A, Bisbocci D, Fonzo D, Gaisano GP and Ceresa F (1975). Effect of synthetic luteinizing hormone releasing hormone (LH-RH) on the release of gonadotropins in Cushing's disease. J Clin Endocrinol Metab **40**: 892-5.

Bomba M, Gambera A, Bonini L, Peroni M, Neri F, Scagliola P and Nacinovich R (2007). Endocrine profiles and neuropsychologic correlatese of functional hypothalamic amenorrhea in adolescents. Fertil Steril **87**(4): 876-85.

Bornstein SR, Webster EL, Torpy DJ, Richman SJ, Mitsiades N, Igel M, Lewis DB, Rice KC, Joost HG, Tsokos M and Chrousos GP (1998). Chronic effect of a nonpeptide corticotropin-releasing hormone type 1 receptor antagonist on pituitary-adrenal function, body weight, and metabolic regulation. Endocrinol **139**(4): 1546-55.

Brinkley HJ (1981). Endocrine signaling and female reproduction. Biol Reprod **24**(1): 22-43.

Briski KP, Vogel KL and McIntryre AR (1995). The antiglucocorticoid, RU486, attenuates stress-induced decreases in plasma-luteinizing hormone concentrations in male rats. Neuroendocrinol **61**(6): 638-45.

Broadbear JH, Winger G, Rivier JE, Rice KC and Woods JH (2002). Corticotropin-releasing hormone antagonists, astressin B and antalarmin: differing profiles of activity in rhesus monkeys. Neuropsychopharmacol **29**: 1112-21.

Bronson FH and Manning JM (1991) The energetic regulation of ovulation: a realistic role for body fat. Biol Reprod **44**: 945-950.

Brundu B, Loucks TL, Adler LJ, Cameron JL and Berga SL (2006). Increased cortisol in the cerebrospinal fluid of women with functional hypothalamic amenorrhea. J Clin Endocrinol Metab **91**: 1561-65.

Bullen BA, Skrinar GS, Beitins IZ, von Mering G, Turnbull BA and McArthur JW (1985). Induction of menstrual disorders by strenuous exercise in untrained women. N Engl J Med **312**: 1349-53.

Butler WR, Malven PV, Willett LB and Bolt DJ (1972). Patterns of pituitary release and cranial output of LH and prolactin in ovariectomized ewes. Endocrinol **91**(3): 793-801.

Cameron JL (1997). Stress and behaviorally induced reproductive dysfunction in primates. Semin Reprod Endocrinol **15**(1): 37-45.

Cameron JL (2000). Reproductive dysfunction in primates, behaviorally induced. In: Fink G, editor. pp 366-372 Encyclopedia of Stress. New York: Academic Press.

Cameron JL (2004). The use of animal models for mechanistic and developmental studies. Paper presented at the NIMH Workshop on the Prevention of Depression in Children and Adolescents, Rockville MD, June 21-22.

Cameron JL, Bacanu S, Coleman K, Dahl RE, Devlin BJ, Rogers J Ryan ND and Williamson DE (2003). Dissociating components of anxious behavior in young rhesus monkeys: A precursor to genetic studies. In: Fear & Anxiety: Benefits of Translational Research pp 251-71 Gorman JM (ed) American Psychiatric Press: Washington, D.C.

Cameron JL, Bethea CL, Berga SL, Herod SM and Centeno ML (2009). The role of serotonin in mediating stress-induced infertility: Studies in a monkey model of functional

hypothalamic amenorrhea. Annual Meeting of the NICHD Specialized Cooperative Center Program in Reproductive Research. Portland, OR.

Cameron JL, Bridges MW, Graham RE, Bench L, Berga SL and Matthews K (1998). Basal heartrate predicts development of reproductive dysfunction in response to psychological stress. Endocrine Society 80th Annual Meeting, Abstract PI-76.

Cameron JL, Bytheway JA, Guay S, Bethea CL, Kerr D, Rockcastle N, Perel JM and Axelson JD (2004). Treatment with a serotonin reuptake inhibitor increases reproductive hormone secretion in stress sensitive monkeys. Society for Neuroscience Annual Meeting, Abstract No. 192.18.

Cameron JL, Helmreich DL and Schreihofer DA (1993). Modulation of reproductive hormone secretion by nutritional intake: stress signals versus metabolic signals. Hum Reprod **8**(Suppl 2): 162-7.

Cameron JL and Nosbisch C (1991). Slowing of pulsatile LH and testosterone secretion during short-term fasting in adult male rhesus monkeys (Macaca mulatta). Endocrinol **128**: 1532-40.

Cameron JL, Weltzin T, McConaha C, Helmreich DL and Kaye WH (1991). Suppression of reproductive axis activity in men undergoing a 48 hour fast. J Clin Endocrinol Metab **73**:35-41.

Cagampang FRA, Maeda K-L, Yokoyama A and Ota K (1990). Effect of food deprivation on the pulsatile LH release in cycling and ovariectomized female rats. Hormone Metab Res **22**:269-272.

Carmel PW, Araki S and Ferin M (1976). Pituitary stalk portal blood collection in rhesus monkeys: evidence for pulsatile release of GnRH. Endocrinol **99**(1): 243-8.

Carroll BJ, Feinberg M, Greden JF, Tarika J, Albala AA, Haskett RF, James NM, Kronfol Z, Lohr N, Steiner M, deVigne JP and Young E (1981). A specific laboratory test for the diagnosis of melancholia: standardization, validation, and clinical utility. Arch Gen Psychiatry **38**(1): 15-22.

Caspi A and Moffitt TE (2006). Gene-environment interactions in psychiatry: joining forces with neuroscience. Nat Rev Neurosci **7**: 583-90.

Catania EH, Pimenta A and Levitt P (2008). Genetic deletion of *Lsamp* causes exaggerated behavioral activation in novel environments. Behav Brain Res **188**: 380-90.

Centeno ML, Reddy AP, Smith LJ, Sanchez RL, Henderson JA, Salli NC, Hess DJ, Pau FK and Bethea CL (2007). Serotonin in microdialysate from the mediobasal hypothalamus increases after progesterone administration to estrogen primed macaques. Eur J Pharmacol **555**(1): 67-75.

Centeno ML, Sanchez RL, Cameron JL and Bethea CL (2007a). Hypothalamic expression of serotonin 1A, 2A and 2C receptor and GAD67 mRNA in female cynomolgus monkeys with different sensitivity to stress. Brain Res **1142**: 1-12.

Centeno ML, Sanchez RL, Cameron JL and Bethea CL (2007b). Hypothalamic gonadotrophin-releasing hormone expression in female monkeys with different sensitivity to stress. J Neuroendocrinol **19**(8): 594-604.

Centeno ML, Sanchez RL, Reddy AP, Cameron JL and Bethea CL (2007c). Corticotropin-releasing hormone and pro-opiomelanocortin gene expression in female monkeys with different sensitivity to stress. Neuroendocrinol **86**(4): 277-88.

Chalmers DT, Lovenberg TW and De Souza EB (1995). Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci **15**: 6340-50.

Chan EC, Falconer J, Madsen G, Rice KC, Webster EL, Chrousos GP and Smith R (1998). A corticotropin-releasing hormone type 1 receptor antagonist delays parturition in sheep. Endocrinol **139**(7): 3357-60.

Chappel SC and Howles C (1991). Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. Hum Reprod **6**: 1206-12.

Charmandari E, Tsigos C and Chrousos G (2005). Endocrinology of the stress response. Annu Rev Physiol **67**: 259-84.

Charney DS (2004). Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress. Am J Psychiatry **161**: 195-216.

Chen YPL. International Patent Number: WO94/13676 June 23, 1994.

Chowdhury GM, Fujioka T and Nakamura S (2000). Induction and adaptation of Fos expression in the rat brain by two types of acute restraint stress. Brain Res Bull **52**(3): 171-82.

Chrousos GP, Torpy DJ and Gold PW (1998). Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. Ann Intern Med **129**(3): 229-40.

Cicchetti D and Rogosch FA (2002). A developmental psychopathology perspective on adolescence. J Consult Clin Psychol **70**: 6-20.

Clarke IJ and Cummins JT (1982). The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinol **111**(5): 1737-9.

Clifton DK, Bremner WJ and Steiner RA (1982). An automated technique for the radiographic determination of bone age. J Med Primatol **11**(3): 147-54.

Cohen LH, Gunthert KC, Butler AC, O'Neill SC and Tolpin LH (2005). Daily affective reactivity as prospective predictor of depressive symptoms. J Pers **73**(6): 1687-713.

Cohen S and Hamrick N (2003). Stable individual differences in physiological response to stressors: implications for stress-elicited changes in immune related health. Brain Behav Immun **17**(6): 407-14.

Cole RL and Sawchenko PE (2002). Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J Neurosci **22**(3): 959-69.

Coleman K, Dahl RE, Ryan ND and Cameron JL (2003). Growth hormone response to growth hormone-releasing hormone and clonidine in young monkeys: correlation with behavioral characteristics. J Child Adolesc Psychopharmacol **13**(3): 227-41.

Collier DA, Stober G, Li T, Heils A, Catalano M, Di Bella D, Arranz MJ, Murray RM, Vallada HP, Bengel D, Muller CR, Roberts GW, Smeraldi E, Kirov G, Sham P and Lesch KP (1996). A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. Mol Psychiatry **1**: 453-60.

Coquelin A and Bronson FH (1980). Secretion of luteinizing hormone in male mice: factors that influence release during sexual encounters. Endocrinol **106**(4): 1224-9.

Coryell W, Noyes R JR and Reich J (1991). The prognostic significance of HPA-axis disturbance in panic disorder: a three-year follow-up. Biol Psychiatry **29**(2): 96-102.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P and Stenzel-Poore MP (2000). Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet **24**(4): 403-9.

Coste SC, Murray SE and Stenzel-Poore MP (2001). Animal models of CRH excess and CRH receptor deficiency display altered adaptations to stress. Peptides **22**(5): 733-41.

Couzinet B, Lahlou N, Lestrat N, Bouchard P, Roger M and Schaison G (1986). Pulsatile luteinizing hormone releasing hormone treatment for induction of ovulation. Radioimmunoassay of plasma LHRH and comparative study of subcutaneous versus intravenous routes of administration. J Endocrinol Invest **9**(2): 103-8.

Couzinet B, Young J, Brailly S, Le Bouc Y, Chanson P and Schaison G (1999). Functional hypothalamic amenorrhea: a partial and reversible gonadotrophin deficiency of nutritional origin. Clin Endocrinol (Oxf) **50**(2): 229-35.

Crane JW, Buller KM and Day TA (2003). Evidence that the bed nucleus of the stria terminalis contributes to the modulation of hypophysiotropic corticotropin

releasing factor cell responses to systemic interleukin-1beta. J Comp Neurol **467**(2): 232-42.

Crowley WF Jr, Filicori M, Spratt DI and Santoro NF (1985). The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res **41**: 473-531.

Crowley WF Jr and McArthur JW (1980). Simulation of the normal menstrual cycle in Kallman's syndrome by pulsatile administration of luteinizing hormone-releasing hormone (LHRH). J Clin Endocrinol Metab **51**(1): 173-5.

Currier GW, Citrome LL, Zimbroff DL, Oren D, Manos G, McQuade R, Pikalov AA and Crandall DT (2007). Intramuscular aripiprazole in the control of agitation. J Psychiatr Pract **13**(3): 159-69.

Dahl RE and Gunnar MR (2009). Heightened stress responsiveness and emotional reactivity during pubertal maturation: implications for psychopathology. Dev Psychopathol **21**(1): 1-6.

Dahlström A and Fuxe K (1964). Localization of monoamines in the lower brain stem. Experientia **20**(7): 398-9.

Davenport MD, Tiefenbacher S, Lutz CK, Novak MA and Meyer JS (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen Comp Endocrinol **147**: 255-61.

De Souza EB (1987). Corticotropin-releasing factor receptors in the rat central nervous system: characterization and regional distribution. J Neurosci **7**(1): 88-100.

Deak T, Nguyen KT, Ehrlich AL, Watkins LR, Spencer RL, Maier SF, Licinio J, Nielsen DM, Carey GJ and Gold LH (2004). Antidepressant-like activity of corticotropin-

releasing factor type-1 receptor antagonists in mice. Eur J Pharmacol **499**(1-2): 135-46.

Del Ser T, McKeith I, Anand R, Cicin-Sain A, Ferrara R and Spiegel R (2000). Dementia with Lewy bodies: findings from an international multicentre study. Int J Geriatr Psychiatry **15**(11): 1034-45.

Dierschke DJ, Battacharya AN, Atkinson LE and Knobil E (1970). Circhoral oscillations of plasma LH levels in the ovariectomized rhesus monkey. Endocrinol **87**(5): 850-3.

Dobson H and Smith RF (2000). What is stress, and how does it affect reproduction? Animal Reprod Science **60**: 743-52.

Dobson H, Ghuman S, Prabhakar S and Smith R (2003). A conceptual model of the influence of stress on female reproduction. Reproduction **125**: 151-63.

Donovan BT, Haar MB and Parvizi N (1977). Gonadotrophin secretion in the ovariectomized guinea pig: effects of electrical stimulation of the hypothalamus and of LHRH. J Physiol **265**(3): 597-613.

Dubey AK, Cameron JL, Steiner RA and Plant TM (1986). Inhibition of gonadotropin secretion in castrated male rhesus monkeys (Macaca mulatta) induced by dietary restriction: analogy with the prepubertal hiatus of gonadotropin release. Endocrinol **118**: 518-525.

Dubey AK and Plant TM (1985). A suppression of gonadotropin secretion by cortisol in castrated male rhesus monkeys (*Macaca mulatta*) mediated by the interruption of hypothalamic gonadotropin-releasing hormone release. Biol Reprod **33**: 423-31.

Dudas B and Merchenthaler I (2004). Close anatomical associations between beta-endorphin and luteinizing hormone releasing hormone neuronal systems in the human diencephalons. Neurosci **124**(1): 221-9.

Dunn AJ and Berridge CW (1990). Physiological and behavioral esponses to corticotropin-releasing factor administratin: is CRF a mediator of aniety or stress responses? Brain Res Rev **15**: 71-100.

Falck B (1959). Site of production of oestrogen in rat ovary as studied in micro-transplants. Acta Physiol Scand Suppl **47**: 1-101.

Fava GA, Trombini G, Grandi S, Bernardi M, Evangelisti LP, Santarsiero G and Orlandi C (1984). Depression and anxiety associated with secondary amenorrhea. Psychosomatics **25**(12): 905-8.

Feldman S and Conforti N (1980). Adrenal responses and neurotransmitters in posterior hypothalamic deafferentation. J Neurosci Res **5**: 19-23.

Feng YJ, Shalts E, Xia LN, Rivier J, Rivier C, Vale W and Ferin M (1991). An inhibitory effects of interleukin-1a on basal gonadotropin release in the ovariectomized rhesus monkey: reversal by a corticotropin-releasing factor antagonist. Endocrinol **128**: 2077-82.

Ferin M (1995). The antireproductive role of corticotropin releasing hormone and interleukin-1 in the female rhesus monkey. Ann Endocrinol (Paris) **56**: 181-6.

Ferin M and Vande Wiele R (1984). Endogenous opioid peptides and the control of the menstrual cycle. Eur J Obstet Gynecol Reprod Biol **18**: 365-73.

Fevold H, Hisaw F and Leonard S (1931). The gonad stimulating and the luteinizing hormones of the anterior lobe of the hypophesis. Am J Physiol **97**: 291-301.

Fink G, Sumner BE, McQueen JK, Wilson H and Rosie R (1998). Sex steroid control of mood, mental state and memory. Clin Exp Pharmacol Physiol **25**(10): 764-75.

Foster DL, Ebling FJP, Micka AF, Vannerson LA, Bucholtz DC, Wood RI, Suttie JM and Fenner DE (1989). Metabolic interfaces between growth and reproduction: Nutritional modulation of gonadotropin, prolactin, and growth hormone secretion in the growth-limited female lamb. Endocrinol **125**: 342-350.

Fox NA (2004). Temperament and early experience form social behavior. Ann N Y Acad Sci **1038**: 171-78.

Foxcroft GR, Pomerantz DK and Nalbandov AV (1975). Effects of estradiol-17beta on LH-RH/FSH-RH-induced, and spontaneous, LH release in prepubertal female pigs. Endocrinol **96**(3): 551-7.

Freeman ME (1994). The neuroendocrine control of the ovarian cycle of the rat. In The Physiology of Reproduction pp 613-658 E Knobil and JD Neill, eds. New York: Raven Press.

French JA, Fite JE, Jensen H, Oparowski K, Rukstalis MR, Fix H, Jones B, Maxwell H, Pacer M, Power ML and Schulkin J (2007). Treatment with the CRH-1 antagonist antalarmin reduces behavioral and endocrine responses to social stressors in marmosets (*Callithrix kuhlii*). Am J Primatol **69**(8): 877-89.

Frisch RE (1984). Body fat, puberty and fertility. Biol Rev **59**: 161-188.

Fuchs E and Flűgge G (2002). Social stress in tree shrews: effects on physiology, brain function, and behavior of subordinate individuals. Pharmacol Biochem Behav **73**(1): 247-58.

Fuller RW (1992). The involvement of serotonin in regulation of pituitary-

adrenocortical function. Front Neuroendocrinol **13**(3): 250-70.

Gay VL and Sheth NA (1972). Evidence for a periodic release of LH in castrated male and female rats. Endocrinol **90**(1): 158-62.

Genazzani AD, Petraglia F, Gastaldi M, Volpogni C, Gamba O and Genazzani AR (1995). Naltrexone treatment restores menstrual cycles in patients with weight loss-related amenorrhea. Fertil Steril **64**: 951-6.

Giles DE and Berga SL (1993). Cognitive and psychiatric correlates of functional hypothalamic amenorrhea: a controlled comparison. Fertil Steril **60**: 486-92.

Goldsmith HH and Rieser-Danner L (1990). Assessing early temperament. In: Handbook of Psychological and Educational Assessment of Children, Vol 2, Personality, Behavior, and Context. pp 345-78 Edited by Reynolds CR, Kamphaus R. New York: Guilford.

Goodman RL and Karsch FJ (1981). The hypothalamic pulse generator: a key determinant of reproductive cycles in sheep. In Biological Clocks in Seasonal Reproductive Cycles pp 223-36 BK Follett and DE Follett, eds. Bristol: John Wiley and Sons, Ltd.

Goodman WK (2004). Selecting pharmacotherapy for generalized anxiety disorder. J Clin Psychiatry **65** Suppl 13: 8-13.

Goodman LR and Warren MP (2005). The female athlete and menstrual function. Curr Opin Obstet Gynecol **17**(5): 466-70.

Grakalic I, Schindler CW, Baumann MH, Rice KC and Riley AL (2006). Effects of stress modulation on morphine-induced conditioned place preferences and plasma

corticosterone levels in Fischer, Lewis, and Sprague-Dawley rat strains. Psychopharmacol (Berl) **189**(3): 277-86.

Greenberg BD, Li Q, Lucas FR, Hu S, Sirota LA, Benjamin J, Lesch KP, Hamer D and Murphy DL (2000). Association between the serotonin transporter promoter polymorphism and personality traits in a primarily female population sample. Am J Med Genet **96**(2): 202-16.

Gunnar MR, Wewerka S, Frenn K, Long JD and Griggs C (2009). Developmental changes in hypothalamus-pituitary-adrenal activity over the transition to adolescence: normative changes and associations with puberty. Dev Psychopathol **21**(1): 69-85.

Guoth J and Zsolnai B (1986). LHRH treatment of hypothalamic amenorrhea. Acta Med Hung **43**(2): 223-8.

Guptarak J, Selvamani A and Uphouse L (2004). GABAA-5-HT1A receptor interaction in the mediobasal hypothalamus. Brain Res 1027(1-2): 144-50.

Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, Gold PW (2000). Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. PNAS **97**(11): 6079-84.

Haeger P, Andres ME, Forray MI, Daza C, Araneda S and Gysling K (2006). Estrogen receptors α and β differentially regulate the transcriptional activity of the urocortin gene. J Neurosci **26**(18): 4908-16.

Hamilton M (1960). A rating scale for depression. J Neurol Neurosurg Psychiat **23**: 56-62.

Hammack SE, Pepin JL, DesMarteau JS, Watkins LR and Maier SF (2003). Corticotropin releasing hormone type 2 receptors in the dorsal raphe nucleus mediate the behavioral consequences of uncontrollable stress. J Neurosci **23**: 1019-25.

Hammack SE, Richey KJ, Schmid MJ, LoPresti ML Watkins LR and Maier SF (2002). The role of corticotropin-releasing hormone in the dorsal raphe nucleus in mediating the behavioral consequences of uncontrollable stress. J Neurosci **22**: 1020-6.

Heath-Lange S, Ha JC and Sackett GP (1999). Behavioral measurement of temperament in male nursery-raised infant macaques and baboons. Am J Primatol **47**(1): 43-50.

Helmreich DL, Mattern LG and Cameron JL (1993). Lack of a role of the hypothalamic-pituitary-adrenal axis in fasting-induced suppression of LH secretion in adult male rhesus monkeys (Macaca mulatta). Endocrinol **132**: 2427-37.

Henderson JA and Bethea CL (2008). Differential effects of ovarian steroids and raloxifene on serotonin 1A and 2C receptor protein expression in macaques. Endocrine [Epub ahead of print].

Herod SM, Centeno ML, Bethea CL and Cameron JL (2008). Evidence for a non-neuroendocrine role of corticotropin-releasing hormone (CRH) in female cynomolgus monkeys sensitive to stress-induced reproductive dysfunction. Abstract No. 283.3, 38th Annual Meeting of the Society for Neuroscience, Washington, D.C.

Herod SM, Dolney R, Bytheway J, Berga SL and Cameron JL (2006). Female monkeys who show stress-induced reproductive dysfunction also have increases in certain forms of anxious behavior. Abstract No. 257.22, 36th Annual Meeting of the Society for Neuroscience, Atlanta, GA.

Hery M, Laplante E and Kordon C (1976). Participation of serotonin in the phasic release of LH. I. Evidence from pharmacological experiments. Endocrinol **99**(2): 496-503.

Hery M, Laplante E and Kordon C (1978). Participation of serotonin in the phasic release of luteinizing hormone. II. Effects of lesions of serotonin-containing pathways in the central nervous system. Endocrinol **102**(4): 1019-25.

Hery M, Semont A, Fache M-P, Faudon M and Hery F (2000). The effects of serotonin on glucocorticoid receptor binding in rat raphe nuclei and hippocampal cells in culture. J Neurochem **74**: 406-13.

Higley JD, Mehlman PT, Poland RE, Taub DM, Vickers J, Suomi SJ and Linnoila M (1996). CSF testosterone and 5-HIAA correlate with different types of aggressive behaviors. Biol Psychiatry **40**(11): 1067-82.

Higley JD, Suomi SJ and Linnoila M (1992). A longitudinal assessment of CSF monoamine metabolite and plasma cortisol concentrations in young rhesus monkeys. Biol Psychiatry **32**(2): 127-45.

Hirono N, Mori E, Tanimukai S, Kazui H, Hashimoto M, Hanihara T and Imamura T (1999). Distinctive neurobehavioral features among neurodegenerative dementias. J Neuropsychiatry Clin Neurosci **11**(4): 498-503.

Hirshfeld DR, Rosenbaum JF, Biederman J, Bolduc EA, Faraone SV, Snidman N, Reznick JS and Kagan J (1992). Stable behavioral inhibition and its association with anxiety disorder. J Am Acad Child Adolesc Psychiatry **31**: 103-11.

Hoffman AR and Crowley WF Jr (1982). Induction of puberty in men by long-term pulsatile administration of low-dose gonadotropin-releasing hormone. N Engl J Med **307**(20): 1237-41.

Holmes A (2008). Genetic variation in cortico-amygdala serotonin function and risk for stress-related disease. Neurosci Biobehav Rev **32**(7): 1293-314.

Hotchkiss J and Knobil E (1994). The menstrual cycle and its neuroendocrine control. In The Physiology of Reproduction pp 711-749 E Knobil and JD Neill, eds. New York: Raven Press.

Hsin LW, Tian X, Webster EL, Coop A, Caldwell TM, Jacobson AE, Chrousos GP, Gold PW, Habib KE, Ayala A, Eckelman WC, Contoreggi C and Rice KC (2002). CHR-R1 receptor binding and lipophilicity of pyrrolopyrimidines, potential nonpeptide corticotropin-releasing hormone type 1 receptor antagonists. Bioorg Med Chem **10**(1): 175-83.

Jabbi M, Korf J, Kema IP, Hartman C, van der Pompe G, Minderaa RB, Ormel J and den Boer JA (2007). Convergent genetic modulation of the endocrine stress response involves polymorphic variations of 5-HTT, COMT, and MAO-A. Mol Psychiatry 12: 483-90.

Jensen JT, Zelinski MB, Stanley JE, Fanton JW and Stouffer RL (2008). The phosphodiesterase 3 inhibitor ORG 9935 inhibits oocyte maturation in the

naturally selected dominant follicle in rhesus macaques. Contraception **77**(4): 303-7.

Jeste DV, Meeks TW, Kim DS and Zubenko GS (2006). Research agenda for DSM-V: diagnostic categories and criteria for neuropsychiatric syndromes in dementia. J Geriatr Psychiatry Neurol **19**(3): 160-71.

Johnson LM and Gay VL (1981). Luteinizing hormone in the cat. I. Tonic secretion. Endocrinol **109**(1): 240-6.

Kalantaridou S, Makrigiannakis A, Zoumakis E and Chrousos GP (2007). Peripheral corticotropin-releasing hormone is produced in the immune and reproductive systems: actions, potential roles and clinical implications. Front Biosci **12**: 572-80.

Kalin NH (2003). Nonhuman primate studies of fear, anxiety, and temperament and the role of benzodiazepine receptors and GABA systems. J Clin Psychiatry **64**(Suppl 3): 41-4.

Kalin NH and Shelton SE (1989). Defensive behaviors in infant rhesus monkeys: environmental cues and neurochemical regulation. Science **243**(4899): 1718-21.

Kalin NH and Shelton SE (2003). Non-human primate models to study anxiety, emotion regulation, and psychopathology. Ann N Y Acad Sci **1008**: 189-200.

Kalin NH, Shelton SE and Takahashi LK (1991). Defensive behaviors in infant rhesus monkeys: ontogeny and context-dependent selective expression. Child Dev **62**(5): 1175-83.

Kalra S, Einarson A, Karaskov T, Van Uum S, Koren G (2007). The relationship between stress and hair cortisol in healthy pregnant women. Clin Invest Med **30**(2): 103-7.

Kamel FA and Kubajak CL (1987). Modulation of gonadotropin secretion by corticosterone: Interaction with gonadal steroids and mechanism of action. Endocrinol **121**: 561-8.

Kant GJ, Bunnel BN, Mougey EH, Pennington LL and Meyerhoff JL (1983). Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats. Pharmacol Biochem Behav **18**(6): 967-71.

Karsch FJ (1980). Twenty-fifth Annual Bowditch Lecture. Seasonal reproduction: a sage of reversible fertility. Physiologist **3**(6): 29-38.

Katongole CB, Naftolin F and Short RV (1971). Relationship between blood levels of luteinizing hormone and testosterone in bulls, and the effects of sexual stimulation. J Endocrinol **50**(3): 457-66.

Katongole CB, Naftolin F and Short RV (1974). Seasonal variations in blood luteinizing hormone and testosterone levels in rams. J Endocrinol **60**(1): 101-6.

Kaufman J, Plotsky PM, Nemeroff CB and Charney DS (2000). Effects of early life adverse experiences on brain structure and function: clinical implication. Biol Psychiatry **48**: 778-90.

Kelly JP, Wrynn AS and Leonard BE (1997). The olfactory bulbectomized rat as a model of depression: an update. Pharmacol Ther **74**(3): 299-316.

Khoury SA, Reame NE, Kelch RP and Marshall JC (1986). Diurnal patterns of pulsatile luteinizing hormone secretion in hypothalamic amenorrhea: Reproducibility and responses to opiate blockade and an α_2-adrenergic agonist. J Clin Endocrinol Metab **64**(4): 755-62.

Kirby LH, Rice K and Valentino RJ (2000). Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. Neuropsychopharnacol **22**: 148-62.

Kirschbaum C, Kudielka BM, Gaab J, Schommer NC and Hellhammer DH (1999). Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. Psychosom Med **61**: 154-62.

Kishimoto T, Radulovic J, Radulovic M, Lin CRH, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG and Spiess J (2000). Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet **24**(4): 415-9.

Knobil E (1980). The neuroendocrine control of the menstrual cycle. Recent Prog Horm Res **36**: 53-88.

Knobil E (1981). Patterns of hypophysiotropic signals and gonadotropin secretion in the rhesus monkey. Biol Reprod **24**(1): 44-9.

Knobil E (1989). The electrophysiology of the GnRH pulse generator in the rhesus monkey. J Steroid Biochem **33**(4): 669-71.

Kobayashi M, Nakano R and Ooshima A (1990). Immunohistochemical localization of pituitary gonadotrophins and gonadal steroids confirms the 'two-cell, two-gonadotrophin' hypothesis of steroidogenesis in the human ovary. J Endocrinol **126**: 483-88.

Kondoh Y, Uemura T, Murase M, Yokoi N, Ishikawa M and Hirahara F (2001). A longitudinal study of disturbances of the hypothalamic-pituitary-adrenal axis in women with progestin-negative functional hypothalamic amenorrhea. Fertil Steril **76**: 748-752.

Koob GF (1999). Corticotropoin-releasing factor, norepinephrine, and stress. Biol Psychiatry **46**(9): 1167-80.

Kottler ML, Hamel A, Malville E and Richard N (2004). GnRH deficiency: new insights from genetics. J Soc Biol **198**(1): 80-7.

Kudielka BM, Schommer NC, Hellhammer DH and Kirschbaum C (2004). Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. Psychoneuroendocrinol **29**(8): 983-92.

Laughlin GA, Dominguez CE and Yen SS (1998). Nutritional and endocrine-metabolic aberrations in women with functional hypothalamic amenorrhea. J Clin Endocrinol Metab **83**: 25-32.

Le AD, Harding S, Juzytsch W, Fletcher PJ and Shaham Y (2002). The role of corticotropin-releasing factor in the median raphe nucleus in relapse to alcohol. J Neurosci **22**(18): 7844-9.

Leal-Cerro A, Gippini A, Amaya MJ, Lage M, Mato JA, Dieguez C and Casanueva FF (2003). Mechanisms underlying the neuroendocrine response to physical exercise. J Endocrinol Invest **26**(9): 879-85.

Lechin F, van der Dijs B and Hernandez-Adrian G (2006). Dorsal raphe vs. median raphe serotonergic antagonism. Anatomical, physiological, behavioral, neuroendocrinological, neropharmacological and clinical evidences: Relevance for neuropharmacological therapy. Prog in Neuro-Psychopharmacol & Biol Psychiatry **30**: 565-85.

Leranth C, MacLusky NJ, Brown TJ, Chen EC, Redmond DE Jr and Naftolin F (1992). Transmitter content and afferent connections of estrogen-sensitive progestin

receptor-containing neurons in the primate hypothalamus. Neuroendocrinol **55**(6): 667-82.

Lesch PK, Bengel D, Heils A, Sabol SZ, Greenberg BG, Petri S, Benjamin J, Muller CR, Hamer DH and Murphy DL (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science **274**: 1527-31.

Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, Arca M, Berto E, Milite G and Marcheggiano A (2000). Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. Amer J of Gastroent **95**(5): 1213-20.

Levine S (2000). Influence of psychological variables on the activity of the hypothalamic-pituitary-adrenal axis. Eur J Pharmacol **48**: 149-60.

Leyendecker G, Wildt L and Hansmann M (1980). Pregnancies following chronic intermittent (pulsatile) administration of Gn-RH by means of a portable pump ("Zyklomat") – a new approach to the treatment of infertility in hypothalamic amenorrhea. J Clin Endocrinol Metab **51**(5): 11214-6.

Lindahl MS, Olovsson M, Nyberg S, Thorsen K, Olsson T and Poromaa IS (2007). Increased cortisol responsivity to adrenocorticotropic hormone and low plasma levels of interleukin-1 receptor antagonist in women with functional hypothalamic amenorrhea. Fertil Steril **87**(1): 136-42.

Lindenmayer JP (2000). The pathophysiology of agitation. J Clin Psychiatry **61**(Suppl 14): 5-10.

Liu JH (1990). Hypothalamic amenorrhea: clinical perspectives, pathophysiology, and management. Am J Obstet Gynecol **163**(5 Pt 2): 1732-6.

Liu JH and Bill AH (2008). Stress-associated or functional hypothalamic amenorrhea in the adolescent. Ann N Y Acad Sci 1135: 179-84.

Loucks AB, Mortola JF, Girton L and Yen SSC (1989). Alterations in the hypothalamic-pituitary-ovarian and hypothalamic-pituitary-adrenal axes in athletic women. J Clin Endocrinol Metab **68**: 402-411.

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J and Shekhar A (2005). Modulation of anxiety circuits by serotonergic systems. Stress **8**(4): 233-46.

Lowry CA, Rodda JE, Lightman SL and Ingram CD (2000). Corticotropin-releasing factor increases in vitro firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for activation of a topographically organized mesolimbocortical serotonergic system. J Neurosci **20**(20): 7728-36.

Luiten PG, ter Horst GJ, Karst H and Steffens AB (1985). The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord. Brain Res **329**: 374-78.

Luton J, Thiebolt P, Valcke J, Mahoudeau JA and Bricaire H (1977). Reversible gonadotropin deficiency in male Cushing's disease. J Clin Endocrinol Metab **45**: 488-95.

Ma QP, Yin GF, Ai MK and Han JS (1991). Serotonergic projections from the nucleus raphe dorsalis to the amygdala in the rat. Neurosci Lett **134**(1): 21-4.

Makino S, Hashimoto K and Gold PW (2002). Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol

Biochem Behav **73**(1): 147-58.

Makrigiannakis A, Zoumakis E, Kalanaridou S, Coutifaris C, Margioris AN, Coukos G, Rice KC, Gravanis A and Chrousos GP (2001). Corticotropin-releasing hormone promotes blastocyst implantation and early maternal tolerance. Nat Immunol **2**(11): 1018-24.

Marcus MD, Loucks TL and Berga SL (2001). Psychological correlates of functional hypothalmic amenorrhea. Fertil Steril **76**(2): 310-16.

McEwen BS (2002). The end of stress as we know it. Joseph Henry Press, Washington DC.

McEwen BS (2008). Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol **583**: 174-85.

McKeith IG (2000). Spectrum of Parkinson's disease, Parkinson's dementia, and Lewy body dementia. Neurol Clin **18**(4): 865-902.

Meczekalski B, Podfigurna-Stopa A, Warenik-Szymankiewicz A and Genazzani AR (2008). Functional hypothalamic amenorrhea: Current view on neuroendocrine aberrations. Gynecol Endocrinol **24**(1): 4-11.

Meczekalski B, Tonetti A, Monteleone P, Bernardi F, Luisi S, Stomati M, Luisi M, Petraglia F and Genazzani AR (2000). Hypothalamic amenorrhea with normal body weight: ACTH, allopregnanolone and cortisol responses to corticotropin-releasing hormone test. Eur J Endocrinol **142**: 280-285.

Mello NK (1988). Effects of alcohol abuse on reproductive function in women. Recent Dev Alcohol **6**: 253-76.

Mello NK, Mendelson JH, Kelly M, Diaz-Migoyo N, Sholar JW (2007). The effects of chronic cocaine self-administration on the menstrual cycle in rhesus monkeys. J Pharmacol Exp Ther **281**(1):70-83

Merriam GR and Wachter KW (1982). Algorithms for the study of episodic hormone secretion. Am J Physiol **237**: R45-R51.

Meyer-Lindenberg A and Weinberger DR (2006). Intermediate phenotypes and genetic mechanisms of psychiatric disorders. Nat Rev Neurosci **7**: 818-27.

Miller DS, Reid RR, Cetel NS, Rebar RW and Yen SSC (1993). Pulsatile administration of low-dose gonadotropin-releasing hormone: Ovulation and pregnancy in women with hypothalamic amenorrhea. JAMA **250**: 2937-41.

Moffitt TE, CAspi A and Rutter M (2005). Strategy for investigating interactions between measured genes and measured environments. Arch Gen Psychiatry **62**: 473-81.

Monzani A, Petraglia F, De Leo V, Fabbri G, D'Ambrogio G, Volpe A and Genazzani AR (1989). Glucocorticoids but not vasopressin or oxytocin inhibit luteinizing hormone secretion in patients with psychogenic amenorrhea. Gynecol Endocrinol **3**: 55-62.

Mosko SS and Jacobs BL (1975). Midbrain raphe neurons: sensitivity to glucocorticoids and ACTH in the rat. Brain Res **89**: 368-75.

Murakami F, Shimomura T, Kotani K, Ikawa S, Nanba E and Adachi K (1999). Anxiety traits associated with a polymorphism in the serotonin transporter gene regulatory region in the Japanese. J Hum Genet **44**(1): 15-17.

Myers DA, Gibson M, Schulkin J and Greenwood Van-Meerveld B (2005). Corticosterone implants to the amygdala and type 1 CRH receptor regulation: effects on behavior and colonic sensitivity. Behav Brain Res **161**(1): 39-44.

Myers KM and Davis M (2007). Mechanisms of fear extinction. Mol Psychiatry **12**(2): 120-50.

Myers LP, Fan R, Zheng Q and Pruett SB (2005). Sodium methyldithiocarbamate causes thymic atrophy by an indirect mechanism of corticosterone upregulation. J Immunotoxicol **2**(2): 97-106.

Nankin HR and Troen P (1971). Repetitive luteinizing hormone elevations in serum of normal man. J Clin Endocrinol Metab **33**(3): 558-60.

Nappi RE, Petraglia F, Genazzani AD, D'Ambrogio G, Zara C and Genazzani AR (1993). Hypothalamic amenorrhea: evidence for a central derangement of hypothalamic-pituitary-adrenal cortex axis activity. Fertil Steril **59**(3): 571-6.

Netter F (1981). Anatomy and blood supply of the suprarenal (adrenal) glands. The Ciba Collection of Medical Illustrations. Vol 4. New York, NY: Ciba Pharmaceutical Company, pp 41-42.

Nordstrom K and Allen MH (2007). Managing the acutely agitated and psychotic patient. CNS Spectr **12**(10 Suppl 17): 5-11.

Norman RL (1994). Corticotropin-releasing hormone effects on luteinizing hormone and cortisol secretion in intact female rhesus macaques. Biol Reprod **50**(4): 949-55.

Norman RL and Smith CJ (1992). Restraint inhibits luteinizing hormone and testosterone secretion in intact male rhesus macaques: effects of concurrent naloxone administration. Neuroendocrinol **55**: 405-15.

Olson BR, Cartledge T, Sebring N, Defensor R and Nieman L (1995) Short-term fasting affects luteinizing hormone secretory dynamics but not reproductive function in normal-weight sedentary women. J Clin Endocrinol Metab **65**: 262-7.

Owens MJ and Nemeroff CV (1991). Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev **43**: 425-73.

Palkovits M, Browstein MJ and Vale W (1985). Distribution of corticotropin-releasing factor in the rat brain. Fed Proc **44**: 215-9.

Patchev VK, Hayashi S, Orikasa C and Almeida OF (1995). Implications of estrogen-dependent brain organization for gender differences in hypothalamic-pituitary-adrenal regulation. FASEB J **9**(5): 419-23.

Pecins-Thompson M, Brown NA and Bethea CL (1998). Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. Brain Res Mol Brain Res **53**(1-2): 120-9.

Pecins-Thompson M, Brown NA, Kohama SG and Bethea CL (1996). Ovarian steroid regulation of tryptophan hydroxylase mRNA expression in rhesus macaques. J Neurosci **16**(21): 7021-9.

Pepperell RJ, De Kretser DM and Burger HG (1975). Studies on the metabolic clearance rate and production rate of human luteinizing hormone and on the initial half-time of its subunits in man. J Clin Invest **56**: 118-26.

Perkins RB, Hall JE and Martin KA (2001). Etiology, previous menstrual function and patterns of neuro-endocrine disturbance as prognostic indicators in hypothalamic amenorrhoea. Hum Reprod **16**: 2198-2205.

Petraglia F, Sutton S, Vale W and Plotsky P (1987). Corticotropin-releasing factor decreases plasma luteinizing hormone levels in female rats by inhibiting gonadotropin-releasing hormone release into hypophysial-portal circulation. Endocrinol **120**(3): 1083-8.

Pimstone B, Epstein S, Hamilton SM, Le Roith D and Hendricks S (1977). Metabolic clearance and plasma half disappearance time of exogenous gonadotropin releasing hormone in normal subjects and in patients with liver disease and chronic renal failure. J Clin Endocrinol Metab **44**(2): 356-60.

Potter E, Sutton S, Donaldson C, Chen R, Perrin M, Lewis K, Sawchenko PE and Vale WW (1994). Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc Natl Acad Sci USA **91**(19): 8777-81.

Plant TM (1983). Ontogeny of gonadotropin secretion in the rhesus macaque (*Macaca mulatta*). In: Norman RL (ed) Neuroendocrine Aspects of Reproduction. New York: Academy Press.

Plant TM, Krey LC, Moossy J, McCormack JT, Hess DL and Knobil E (1978). The arcuate nucleus and the control of gonadotropin and prolactin secretion in the female rhesus monkey (*Macaca mulatta*). Endocrinol **102**(1): 52-62.

Plotsky PM, Owens MJ and Nemeroff CB (1998). Psychoneuroendocrinology of depression: Hypothalamic-pituitary-adrenal axis. Psychiatr Clin North Am **21**: 293-307.

Pohl CR and Knobil E (1982). The role of the central nervous system in the control of ovarian function in higher primates. Annu Rev Physiol **44**: 583-93.

Pohl CR, Richardson DW, Hutchison JS, Germak JA and Knobil E (1983). Hypophysiotropic signal frequency and the functioning of the pituitary-ovarian system in the rhesus monkey. Endocrinol **112**(6): 2076-80.

Price ML, Curtis AL, Kirby LG, Valentino RJ and Lucki I (1998). Effects of corticotropin-releasing factor on brain serotonergic activity. Neuropsychopharmacol **18**: 492-502.

Price ML and Lucki I (2001). Regulation of serotonin releasei n the lateral septum and striatum by corticotropin-releasing factor. J Neurosci **21**: 2833-41.

Puder BA and Papka RE (2001). Hypothalamic paraventricular axons projecting to the female rat lumbosacral spinal cord contain oxytocin immunoreactivity. J Neurosci Res **64**(1): 53-60.

Raap DK, DonCarlos LL, Garcia F, Zhang Y, Muma NA, Battaglia G and Van de Kar LD (2002). Ovariectomy-induced increases in hypothalamic serotonin-1A receptor function in rats are prevented by estradiol. Neuroendocrinol **76**(6): 348-56.

Rahe CH, Owens RE, Gleeger JL, Newton JH and Harms PG (1980). Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinol **107**(2): 498-503.

Rasmussen DD, Liu JH, Wolf PL andYen SSC (1986). Gonadotropin-releasing hormone neurosecretion in the human hypothalamus: *In vitro* regulation by dopamine. J Clin Endocrinol Metab **62**:479-483.

Reame NE, Sauder SE, Case GD, Kelch RP and Marshall JC (1985). Pulsatile gonadotropin secretion in women with hypothalamic amenorrhea: Evidence that

reduced frequency of GnRH secretion is the mechanism of persistent anovulation. J Clin Endocrinol Metab **61**: 851-858.

Reifenstein EC Jr (1946). Psychogenic or "hypothalamic" amenorrhea. Med Clin North Am 30:1103-14.

Reindollar RH, Novak M, Tho SP and McDonough PG (1986). Adult-onset amenorrhea: a study of 262 patients. Am J Obstet Gynecol **155**: 531-43.

Resch M, Scendi G and Haasz P (2004). Bulimia from a gynecological view: hormonal changes. J Obstet Gynaecol **24**(8): 907-10.

Reyes BA, Valentino RJ, Xu G, Van Bockstaele EJ (2005). Hypothalamic projections to locus coeruleus neurons in rat brain. Eur J Neurosci **22**(1): 93-106.

Ringstrom SJ and Schwartz NB (1985). Cortisol suppresses the LH, but not the FSH, response to gonadotropin-releasing hormone after orchidectomy. Endocrinol **116**: 472-4.

Rivest S and Rivier C (1995). The role of corticotrophin-releasing factor and interleukin-1 in the regulation of neurons controlling reproductive functions. Endocr Rev **16**: 177-99.

Rivier C and Rivest S (1991). Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol Reprod **45**(4): 523-32.

Rivier C, Rivier J and Vale W (1986). Stress-induced inhibition of reproductive functions: Role of endogenous corticotropin-releasing factor. Science **231**: 607-9.

Rogers CJ, Brissette-Storkus CS, Chambers WH and Cameron JL (1999). Acute stress impairs NK cell adhesion and cytotoxicity through CD2, but not LFA-1. J Neuroimmunol **99**(2): 230-41.

Root A, DeCherney A, Russ D, Duckett G, Garcia CR and Wallach E (1972). Episodic secretion of luteinizing and follicle stimulating hormones in agonadal and hypogonadal adolescents and adults. J Clin Endocrinol **35**(5): 700-4.

Rosmond R, Dallman MF and Björntorp P (1998). Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. J Clin Endocrinol Metab **83**(6): 1853-9.

Rowe PH, Hopkinson CRN, Shenton JC and Glover TD (1975). The secretion of LH and testosterone in the rabbit. Steroids **25**(3): 313-21.

Roy BN, Reid RL and Van Vugt DA (1999). The effects of estrogen and progesterone on corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid levels in the paraventricular nucleus and supraoptic nucleus of the rhesus monkey. Endocrinol **140**(5): 2191-8.

Sabatini MJ, Ebert P, Lewis DA, Levitt P, Cameron JL and Mirnics K (2007). Amygdala gene expression correlates of social behavior in monkeys experiencing maternal separation. J Neurosci **27**(12): 3295-304.

Sachs GS (2006). A review of agitation in mental illness: burden of illness and underlying pathology. J Clin Psychiatry **67** (Suppl 10): 5-12.

Sakanaka M, Shibassaki T and Lederes K (1987). Corticotropin-releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxide-diaminobenzidene method. J Comp Neurol **260**: 256-98.

Saleh TM and Connell BJ (2003). 17beta-estradiol modulates baroreflex sensitivity and autonomic tone of female rats. J Auton Nerv Syst **80**(3): 148-61.

Samochowiec J, Hajduk A, Samochowiec A, Horodnicki J, Stepien G, Grzywacz A and Kucharska-Mazur J (2004). Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum. Psychiatry Res **128**(1): 21-6.

Sanchez RL, Reddy AP, Centeno ML, Henderson JA and Bethea CL (2005). A second tryptophan hydroxylase isoform, TPH-2 mRNA, is increased by ovarian steroids in the raphe region of macaques. Mol Brain Res **135**: 194-203.

Santen RJ and Bardin CW (1973). Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms. J Clin Invest **52**(10): 217-28.

Sapolsky RM (1985). Stress-induced suppression of testicular function in the wild baboon: Role of glucocorticoids. Endocrinol **116**: 2273-8.

Sapolsky RM and Krey LC (1988). Stress-induced suppression of luteinizing hormone concentrations in wild baboons: role of opiates. J Clin Endocrinol Metab **66**:722-726.

Sauvé B, Koren G, Walsh G, Tokmakejian S and Van Uum SH (2007). Measurement of cortisol in human hair as a biomarker of systemic exposure. Clin Invest Med **30**(5): 183-91.

Sawchenko PE and Swanson LW (1982). Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol **205**: 260-72.

Schreihofer DA, Renda F and Cameron JL (1996). Feeding-inducced stimulation of luteinizing hormone secretion in male rhesus monkeys is not dependent on a rise in blood glucose concentration. Endocrinol **137**(9): 3770-6.

Schulkin J (2006). Angst and the amygdala. Dialogues Clin Neurosci **8**(4): 407-16.

Schutzer WE and Bethea CL (1997). Lack of ovarian steroid hormone regulation of norepinephrine transporter mRNA expression in the non-human primate locus coeruleus. Psychoneuroendocrinol **22**(5): 325-36.

Shalev AY (2002). Acute stress reactions in adults. Biol Psychiatry **51**(7): 532-43.

Shively CA, Grant KA, Ehrenkaufer RL, Mach RH and Nader MA (1997). Social stress, depression, and brain dopamine in female cynomolgus monkeys. Ann N Y Acad Sci **807**: 574-7.

Shively CA, Laber-Laird K and Anton RF (1997). Behavior and physiology of social stress and depression in female cynomolgus monkeys. Biol Psychiatry **41**(8): 871-82.

Shively CA, Mirkes SJ, Lu NZ, Henderson JA and Bethea CL (2003). Soy and social stress affect serotonin neurotransmission in primates. Pharmacogenomics J **3**(2): 114-21.

Shively CA, Register TC, Friedman DP, Morgan TM, Thompson J and Lanier T (2005). Social stress-associated depression in adult female cynomolgus monkeys (Macaca fascicularis). Biol Psychol **69**(1): 67-84.

Shoham Z, Jacobs HS and Insler V (1993). Luteinizing hormone: its role, mechanism of action, and detrimental effects when hypersecreted during the follicular phase. Fertil Steril **59**: 1153-61.

Shroff H, Reba L, Thornton LM, Tozzi F, Klump KL, Berrettini WH, et al (2006). Features associated with excessive exercise in women with eating disorders. Int J Eat Disord **39**(6): 454-61.

Smith ER, Johnson J, Weick RF, Levine S and Davidson JM (1971). Inhibition of the reproductive system in immature rats by inctracerebral implantation of cortisol. Neuroendocrinol **8**: 94-106.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W and Lee KF (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron **20**(6): 1093-102.

Smith TE, French JA (1997). Psychosocial stress and urinary cortisol excretion in marmoset monkeys (*Callithrix kuhli*). Physiol Behav **62**(2): 225-32.

Smith RF, Ghuman SP, Evans NP, Karsch FJ and Dobson H (2003). Stress and the control of LH secretion in the ewe. Reprod Suppl **61**: 267-82.

Smith MJ and Jennes L (2001). Neural signals that regulate GnRH neurons directly during the oestrous cycle. Reprod **122**: 1-10.

Speroff L, Glass RH and Kase NG (1999). Clinical gynecologic endocrinology and infertility. 6th ed. Philadelphia: Lippincott, Williams and Wilkins.

Steckler T and Holsboer F (1999). Corticotropin-releasing hormone receptor subtypes and emotion. Biol Psychiatry **46**: 1480-1508.

Steimer T, la Fleur S and Schulz PE (1997). Neuroendocrine correlates of emotional reactivity and coping in male rats from the Roman high (RHA/Verh)- and low (RLA/Verh)-avoidance lines. Behav Genet **27**(6): 503-12.

Steinbusch HW (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat – cell bodies and terminals. Neurosci **6**(4): 557-618.

Steiner RA and Cameron JL (1989). Endocrine control of reproduction. In *Textbook of Physiology* pp 1289-1342 HD Patton, AF Fuchs, B Hille, AM Scher, and RA Steiner, eds. Philadelphia: W.B. Saunders.

Strekalova T, Spanagel R, Dolgov O and Bartsch D (2005). Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. Behav Pharmacol **16**(3): 171-80.

Suh BY, Liu JH, Berga SL, Quigley ME, Laughlin GA and Yen SS (1988). Hypercortisolism in patients with functional hypothalamic-amenorrhea. J Clin Endocrinol Metab **66**(4): 733-9.

Swanson LW (1987). The hypothalamus. In *Handbook of Chemical Neuroanatomy, Vol. 5, Part I, Integrated Systems of the CNS*. pp.1-124 Björklund A, Hökfelt T and Swanson LW, eds. Amsterdam: Elsevier.

Swanson LW and Kuypers HG (1980). The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. J Comp Neurol **194**(3): 555-70.

Swanson LW, Sawchenko PE, Rivier J and Vale WW (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinol **36**: 165-86.

Takahashi K, Totsune K, Masahiko S, Osamu M, Satoh F, Arihara Z, Sasano H, Iino K and Mouri T (1998). Regional distribution of urocortin-like immunoreactivity and expression of urocortin mRNA in the human brain. Peptides **19**(4): 643-7.

Talge NM, Donzella B and Gunnar MR (2008). Fearful temperament and stress reactivity among preschool-aged children. Infant Child Dev **17**(4): 427-45.

Tapper CM, Naftolin F and Brown-Grant K (1972). Influence of the reproductive state at the time of operation on the early response to ovariectomy in the rat. J Endocrinol **53**: 47-57.

Taylor JA, Goubillon ML, Broad KD and Robinson JE (2007). Steroid control of gonadotropin-releasing hormone secretion: associated changes in pro-opiomelanocortin and preproenkephalin messenger RNA expression in the ovine hypothalamus. Biol Reprod **76**(3): 524-31.

Tersman Z, Collins A and Eneroth P (1991). Cardiovascular responses to psychological and physiological stressors during the menstrual cycle. Psychosom Med **53**:185-97.

Tilbrook AJ, Turner AI and Clarke IJ (2002). Stress and reproduction: Central mechanisms and sex differences in non-rodent species. Stress **5**(2): 83-100.

Tillet Y (1995). Distribution of neurotransmitters in the sheep brain. J Reprod Fertil **Suppl 49**: 199-220.

Timple P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F and Wurst W (1998). Impaired stress response and reduced anxiety in mice lacking a functional corticoropin-releasing hormone receptor 1. Nat Genet **19**(2): 162-6.

Treiber FA, Kamarck T, Schneiderman N, Sheffield D, Kapuku G and Taylor T (2003). Cardiovascular reactivity and development of preclinical and clinical disease states. Psychosom Med **65**(1): 46-62.

Tyrer P and Baldwin D (2006). Generalised anxiety disorder. Lancet **368**(9553): 2156-66.

Tyrka AR, Wier LM, Price LH, Rikhye K, Ross NS, Anderson GM, Wilkinson CW and Carpenter LL (2008). Cortisol and ACTH responses to the Dex/CRH test: influence of temperament. Horm Behav **53**(4): 518-25.

Ule G, Schwechheimer K and Bauer M (1984). On the ultrastructure and immunocytology of arcuate neurons with perpetuated feedback effect. Clin Neuropathol **3**(1): 28-31.

Vale W, Spiess J, Rivier C and Rivier J (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science **213**(4514): 1394-7.

Vale W, Vaughan J and Perrin M (1997). Corticotropin-releasing factor (CRF) family of ligands and their receptors. The Endocrinologist **7**: 3S-9.

Valentino RJ, Foote SL and Page ME (1993). The locus coeruleus as a site for integrating corticotropin-releasing factor and noradrenergic meidation of stress responses. Ann NY Acad Sci **697**: 173-88.

Valentino RJ, Liouterman L and Van Bockstaele EJ (2001). Evidence for regional heterogeneity in corticotropin-releasing factor interactions in the dorsal raphe nucleus. J Comp Neurol **435**: 450-63.

Veldhuis JD, O'Dea LSL and Johnson ML (1989). The nature of the gonadotropin-releasing hormone stimulus-luteinizing hormone secretory response of human gonadotrophs in vivo. J Clin Endocrin Metab **68**(3): 661-70.

Vigersky RA, Andersen AE, Thompson RH and Loriaux DL (1997). Hypothalamic dysfunction in secondary amenorrhea associated with simple weight loss. N Eng J Med **297**: 1141-1145.

Vogeser M, Engelhardt D and Jacob K (2000). Comparison of two automated adrenocorticotropic hormone assays. Clin Chem **46**(12): 1998-2000.

von Borrell E, Dobson H and Prunier A (2007). Stress, behaviour and reproductive performance in female cattle and pigs. Horm Behav **52**: 130-8.

Wade GN and Schneider JE (1992). Metabolic fuels and reproductive function in female mammals. Neurosci Biobehav Rev **16**:235-272.

Walker CD, Toufexis DJ and Burlet A (2001). Hypothalamic and limbic expression of CRF and vasopressin during lactation: implications for the control of ACTH secretion and stress hyporesponsiveness. Prog Brain Res **133**: 99-110.

Warren MP and Fried JL (2001). Hypothalamic amenorrhea - The effects of environmental stresses on the reproductive system: a central effect of the central nervous system. Endocrinol Metab Clin North Am **30**: 611-629.

Warren MP and Vande Wiele RI (1973). Clinical and metabolic features of anorexia nervosa. Am J Obstet Gynecol **117**: 435-49.

Warren MP, Voussoughian F, Geer EB, Hyle EP, Adberg CL and Ramos RH (1999). Functional hypothalamic amenorrhea: hypoleptinemia and disordered eating. J Clin Endocrinol Metab **84**: 873-7.

Webster EL, Lewis DB, Torpy DJ, Zachman EK, Rice KC and Chrousos GP (1996). In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinol **137**(12): 5747-50.

Wildt L, Marshall G and Knobil E (1980). Experimental induction of puberty in the infantile female rhesus monkey. Science **207**(4437): 1373-5.

Williams CL, Nishihara M, Thalabard JC, Grosser PM, Hotchkiss J and Knobil E (1990). Corticotropin-releasing factor and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey. Neuroendocrinol **52**: 133-7.

Williams NI, Berga SL and Cameron JL (2007). Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys. *Am* J Physiol Endocrinol Metab **293**(1): 270-6.

Williams NI, Caston-Balderrama AL, Helmreich DL, Parfitt D, Nosbisch C and Cameron JL (2001a). Induction of menstrual cycle disturbances in cynomolgus monkeys during strenuous exercise training: Longitudinal changes in reproductive hormones and menstrual cyclicity. Endocrinol **142**: 2381-9.

Williams NI, Helmreich DL, Parfitt DB, Caston-Balderrama A and Cameron JL (2001b). Evidence for a causal role of low energy availability in the induction of menstrual cycle disturbances during strenuous exercise training. J Clin Endocrinol Metab **86**(11): 5184-93.

Williams NI, Helmreich DL, Parfitt DB, Caston-Balderrama A and Cameron JL (1991). Longitudinal changes in reproductive hormones and menstrual cyclicity in cynomolgus monkeys during strenuous exercise training: abrupt transition to exercise-induced amenorrhea. Endocrinol **142**: 2381-9.

Williamson DE, Coleman K, Bacanu SA, Devlin BJ, Rogers J, Ryan ND and Cameron JL (2003). Heritability of fearful-anxious endophenotypes in infant rhesus macaques: a preliminary report. Biol Psychiatry **53**(4): 284-91.

Wilson RC, Kesner JS, Kaufman JM, Uemura T, Akema T and Knobil E (1984). Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinol **39**: 256-60.

Wilson SC and Sharp PJ (1975). Episodic release of luteinizing hormone in the domestic fowl. J Endocrinol **4**(1): 77-86.

Winslow JT (2005). Neuropeptides and nonhuman primate social deficits associated with pathogenic rearing conditions. Int J Dev Neurosci **23**: 245-51.

Wong ML, Webster EL, Spokes H, Phu P, Ehrhart-Bornstein M, Bornstein S, Park CS, Rice KC, Chrousos GP, Licinio J and Gold PW (1999). Chronic administration of the non-peptide CRH type 1 receptor antagonist antalarmin does not blunt hypothalamic-pituitary-adrenal axis responses to acute immobilization stress. Life Sci **65**(4): PL53-8.

Wu FCW, Taylor PL and Sellar RE (1989). LHRH pulse frequency in normal and infertile men. J Endocrinol **123**: 149-58.

Xiao EN and Ferin M (1988). The inhibitory action of corticotropin-releasing hormone on gonadotropin secretion in the ovariectomized rhesus monkey is not mediated by adrenocorticotropin hormone. Biol Reprod **38**: 763-7.

Xiao EN, Luckhaus J, Niemann W and Ferin M (1989). Acute inhibition of gonadotropin secretion by corticotropin-releasing hormone in the primate: are the adrenal glands involved? Endocrinol **124**(4): 1632-7.

Xiao EN, Xia-Zhang L and Ferin M (1999). Stress and the menstrual cycle: short- and long-term response to a five-day endotoxin challenge during the luteal phase in the rhesus monkey. J Clin Endocrinol Metab **84**: 623-6.

Xiao EN, Xia-Zhang L, Barth A, Zhu J and Ferin M (1998). Stress and the menstrual cycle: relevance of cycle quality in the short- and long-term response to a 5-day endotoxin challenge during the follicular phase in the rhesus monkey. J Clin Endocrinol Metab **83**: 2454-60.

Yen SS, Tsai CC, Naftolin F, Vandenberg G and Ajabor L (1972). Pulsatile patterns of gonadotropin release in subjects with and without ovarian function. J Clin Endocrinol Metab **34**(4): 671-5.

Yen SS, Tsai CC, Vandenberg G and Rebar R (1972). Gonadotropin dynamics in patients with gonadal dysgenesis: a model for the study of gonadotropin regulation. J Clin Endocrinol Metab **35**(6): 897-904.

Zvolensky MJ and Leen-Feldner EW (2005). Anxiety and stress-vulnerability and substance problems: theory, empirical evidence, and directions for future research. Clin Psychol Rev **25**(6): 707-12.