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Neurobiology of Stress-Induced Reproductive Dysfunction in Female Macaques

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Abstract It is now well accepted that stress can precipitate mental and physical illness. However, it is becoming clear that given the same stress, some individuals are very vulnerable and will succumb to illness while others are more resilient and cope effectively, rather than becoming ill. This difference between individuals is called stress sensitivity. Stress sensitivity of an individual appears to be influenced by genetically inherited factors, early life (even prenatal) stress, and by the presence or absence of factors that provide protection from stress. In comparison to other stress-related diseases, the concept of sensitivity versus resilience to stress-induced reproductive dysfunction has received relatively little attention. The studies presented herein were undertaken to begin to identify stable characteristics and the neural underpinnings of individuals with sensitivity to stress-induced reproductive dysfunction. Female cynomolgus macaques with normal menstrual cycles either stop ovulating (stress sensitive) or to continue to ovulate (stress resilient) upon exposure to a combined metabolic and psychosocial stress. However, even in the absence of stress, the stress-sensitive animals have lower secretion of the ovarian steroids, estrogen and progesterone, have higher heart rates, have lower serotonin function, have fewer serotonin neurons and lower expression of pivotal serotonin-related genes, have lower expression of 5HT2A and 2C genes in the hypothalamus, have higher gene expression of GAD67 and CRH in the hypothalamus, and have reduced gonadotropin-releasing hormone transport to the anterior pituitary. Altogether, the results suggest that the neurobiology of reproductive circuits in stress-sensitive individuals is compromised. We speculate that with the application of stress, the dysfunction of these neural systems becomes exacerbated and reproductive function ceases.

Keywords Reproduction · Stress · Serotonin · Corticotropin-releasing hormone · Pro-opiomelanocortin · Beta-endorphin · Paraventricular nucleus · Thalamus · Amygdala · Cynomolgus macaque

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Overview

Exposure to stressful stimuli can lead to a variety of secondary diseases such as anxiety, depression, cardiovascular disease, and immune suppression [1, 2]. Reproductive dysfunction has been recently added to this growing list of stress-related disorders [3]. A significant body of literature has focused upon the application of stress and its

consequences on reproductive cyclicity and the related neuroendocrinology. Early in the 1970s, it was recognized that the stress of population density inhibited estrous cycles in mice [4], and a great deal of effort has been devoted to understanding the effects of maternal stress during pregnancy on offspring physiology and behavior in rodent species [5–7].

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are the gonadotropins that drive ovarian function, estrogen (E) and progesterone secretion (P), menstrual or estrous cyclicity, and ultimately ovulation. In ovariectomized animals, LH secretion becomes elevated and pulsatile. To understand the effect of stress on LH secretion, the ovariectomized pulsatile secretory mode has been utilized. Restraint stress, or activation of the corticotropin-releasing hormone (CRH) receptor type 2 with intracerebroventricular urocortin, suppressed luteinizing hormone pulses in ovariectomized rats [8]. These effects may be mediated via the raphe serotonin system [9–12] or brainstem noradrenergic systems [13, 14], as well as via hypothalamic circuits [15, 16].

Stress and reproduction are important factors in the farming industry. Stresses such as fever, lameness, and transportation can significantly decrease fertility in cows and sheep [17]. Modeling of stress in ewes with endotoxin has enabled the analysis of the neural pathways mediating stress-induced suppression of ovulation. Evidence has been well reviewed indicating that the balance of numerous neurotransmitters such as norepinephrine, serotonin, glutamate, and gamma-aminobutyric acid (GABA), and the neuropeptides CRH, arginine vasopressin (AVP), and neuropeptide Y, impinges directly or indirectly on gonadotropin-releasing hormone (GnRH) neurons to activate or suppress their function depending on the environment [16, 18–20]. AVP plays a greater role that CRH in sheep, but the reverse is true in rodents [16]. Moreover, the medial preoptic region in sheep and rodents contains pivotal GnRH neurons that are not found in humans or primates.

Extreme exercise is considered to be a metabolic stress, and with the advent of greater participation of women in sports, reports emerged that intense athletic participation disrupted menstrual cycles [21]. Further study indicated that there was a suppression of estrogen secretion during the follicular phase and less progesterone secretion during the luteal phase and blunted FSH secretion during the follicular luteal transition in recreational women runners [22].

A clinical syndrome called functional hypothalamic amenorrhea (FHA), characterized by menstrual cycle abnormalities and infertility, is found in a proportion of women who present at infertility clinics [23, 24]. Research indicates that FHA occurs in women with combined moderate psychological and metabolic stress [25] and eating disorders are also common in this population [26]. Moreover, new treatment therapies for FHA that both target strategies for coping with psychological stress and removal of metabolic stresses look very promising [27].

Stress models in non-human primates have employed endotoxin administration [28, 29], interleukin-1 administration [30, 31], CRH administration [32], exercise [33, 34], diet [35], psychosocial stress (relocation to a new room with new neighbors), [36] or combinations of these stresses as found in FHA [37, 38]. Administration of endotoxin, interleukin-1, or CRH activated the hypothalamic–pituitary–adrenal axis, increased cortisol secretion, and suppressed LH and FSH secretion, which could be reversed with the administration of a CRH antagonist [39] or the opiate antagonist, naloxone [40]. A recent study with rhesus monkeys employing the combination of surgery and relocation showed that inadequate LH and progesterone secretion during the luteal phase is the initial defect leading to abnormal menstrual cycle parameters. This study suggested that secretory inadequacy of the corpus luteum represents the first clinical stage in the damage that stress inflicts on the normal menstrual cycle [41].

A pivotal factor in many studies is that stress was applied and results were obtained in a fashion suggesting that all animals respond equally to the stress. However, it is now becoming apparent that certain individuals are more sensitive to stress than others. This is clearly evident in human populations where some individuals succumb to psychiatric and somatic disease after trauma or stress, but other individuals thrive. In animal models, similar results have been obtained by selective breeding in which stress-sensitive and stress-resilient lines are produced [42–45]. Our group has used cynomolgus monkeys and a combination of diet, exercise and relocation to study the effects of stress on reproductive function. When this paradigm is applied to small populations of monkeys, we observed individual differences in reproductive dysfunction with stress. This chapter reviews our investigations and shows that the activity or gene expression in neural circuits mediating stress and reproduction are significantly different in stress-sensitive and stress-resilient individuals *in the absence of stress*.

The Model

Introduction

In many areas of medicine, it is recognized that there are striking individual differences in sensitivity to stress, in that some individuals show marked physiological responses to stressful stimuli and are prone to the development of diseases that occur secondary to chronic stress exposure

(i.e., anxiety, depression, cardiovascular disease, immune suppression), while others are stress resilient and show less physiological response to stressful stimuli and are less likely to develop diseases secondary to chronic stress exposure. Stress sensitivity of an individual appears to be influenced by genetically inherited factors, prior stress exposure (particularly stress exposure in prenatal or early post-natal development), and by the presence or absence of factors that provide protection from stress [1]. In comparison to other stress-related diseases, the concept of sensitivity versus resilience to stress-induced reproductive dysfunction has received relatively little attention to date. However, a comprehensive review of the effects of psychosocial stresses on reproductive function in humans and non-human primates suggests that a number of factors can influence the sensitivity of the reproductive axis to psychosocial stresses, including the perception of stress, the magnitude and duration of stress, social status, and the level of activity within the reproductive axis prior to stress exposure [3]. In addition, several studies also documented individual differences in sensitivity of the reproductive axis to immune stresses [29].

We have undertaken a series of studies in which female cynomolgus macaques were exposed to a combination stress paradigm and their reproductive function was monitored. We found marked differences between individuals in the response of the reproductive system to stress. Following the in vivo characterization, postmortem studies of brain function were executed. These studies revealed that pivotal neural systems in the brain that are involved in stress responsivity were altered in stress-sensitive individuals. Following are studies describing the model, the in vivo characterization, and the postmortem analysis of the brains of animals with differential sensitivity to stress.

Methods

Animals

All studies were reviewed and approved by the Institutional Animal Care and Use Committee of the ONPRC and performed according to federal guidelines. Fifteen adult female cynomolgus monkeys (*Macaca fascicularis*) were housed in single stainless steel cages in a temperature-controlled room (24±2°C), with lights on for 12 h a day (0700–1900 h). Monkeys were imported in 1993 and approximate ages established by dental examination. At the time of this study, the monkeys were 11–14 years of age. Monkeys were provided with two meals a day at 0930 h and 1500 h. At each meal they received six high protein monkey chow biscuits (no. 5047, jumbo biscuits, Ralston Purina Co., St. Louis, MO, USA; approximately 16.5 g each, 3.11 metabolizable Cal/g, 308 Cal/meal). In addition, one-quarter apple was provided with the morning meal. Water was available ad libitum. Animals also received non-caloric treats (ice cubes) and toys in their cages, as well as occasional access to television viewing, as part of the Oregon National Primate Research Center (ONPRC) primate enrichment program. Monkeys had been adapted to these conditions for 2 years prior to the initiation of this study.

Blood Sample Collection

Blood samples for the measurement of serum estradiol and progesterone were collected from unanesthetized animals every day before the animals were exercised. For collection of blood samples, each monkey was trained to jump from its cage into a transport box and enter a specially designed cage that allowed immobilization of the monkey's leg, so a blood sample could be obtained from the femoral region by venipuncture, using previously published techniques [33, 34]. Blood was collected into sterile syringes, transferred into glass tubes, and allowed to clot. Samples were then centrifuged at 2,500 rpm for 10 min, and serum was collected and stored at −20°C in plastic vials until assays were performed. Every 4 weeks, hematocrit was measured. Hematocrits were maintained within the normal range in all monkeys throughout the study. Monkeys were weighed each day at the time of blood sample collection. Hormone assays were conducted as previously described [38].

Monitoring Reproductive Function

Before the study, all animals were accustomed to blood sampling procedures and daily checks for menses, which involved swabbing the vaginal area with a cotton-tipped applicator. The occurrence of several normal menstrual cycles was documented in each monkey before the initiation of the study. The first day of menses was designated the first day of the menstrual cycle. A menstrual cycle was considered normal if it was 25–38 days in length and exhibited typical cyclic changes in reproductive hormones, including a midcycle rise in circulating estradiol followed by a rise in serum progesterone concentrations to levels greater than 2 ng/ml. A monkey was considered to be amenorrheic if she had a cycle longer than 38 days that also showed no evidence of cyclic rises in estradiol and progesterone.

Exercise Training

Animals were trained to run on standard human size treadmills (model 910e, Precor, Inc., Bothell, WA, USA), using previously published techniques [33, 34]. Each treadmill was covered by a Plexiglass box, which had

numerous air holes in the front and back panels to allow adequate ventilation. Monkeys were slowly adapted to the treadmill in the "learn-to-run" menstrual cycle by first being allowed to sit on the treadmill and explore it for several days and then being allowed to walk slowly. After about 1 week of walking, monkeys were given a "max" test to establish the maximum rate which they were capable of running [37]. In the max test, monkeys started running at 0.8 miles/h and speed was then increased 0.2 miles/h every 2 min until the monkey failed to be able to keep up with the pace of the treadmill. Our previous studies showed that monkeys reached maximum heart rate by the time they reached maximum speed [37].

Experimental Design

The experimental model used a combined stress that encompassed mild psychosocial stress + moderate dieting + moderate exercise. The mild psychosocial stress involved moving single-caged monkeys to a new housing room, where they were surrounded by unfamiliar animals. The moderate diet was a 20% decrease in calorie intake, and the moderate exercise was provided by running monkeys on a motorized treadmill at 80% maximum speed (determined for each monkey in the first week of the study) for 1 h per day, 5 days per week.

The initial study involved a five menstrual cycle design (see Fig. 1): *cycle 1*—a control menstrual cycle in which blood samples were collected daily to track reproductive hormone secretion; *cycle 2*—a learn-to-run cycle in which monkeys were accustomed to the treadmill (first sitting on it, and then walking) while blood sample collection was continued; *cycle 3*—stress cycle 1, in which monkeys were moved to a new room on day 1 of the menstrual cycle, calorie intake was decreased by 20% and monkeys initiated running 5 days a week; *cycle 4*—stress cycle 2, in which monkeys moved to a second new room on day 1 and calorie restriction and running were continued; and *cycle 5*—a recovery cycle in which monkeys were moved back to their home environment, food intake was increased back to ad libitum and exercise was terminated. For monkeys that failed to have a menstrual cycle after initiation of the stress, *cycle 3* was continued for 60 days and then the recovery cycle was initiated. For monkeys that failed mense at the end of a second stress cycle, *cycle 4* was continued for 60 days and then the recovery cycle was initiated.

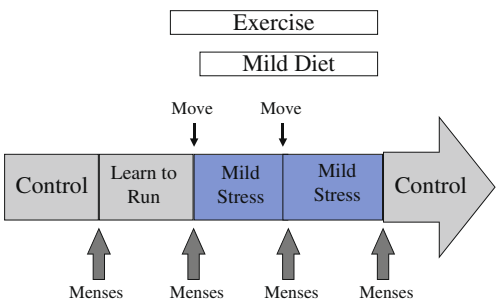

Fig. 1 Schematic diagram of experimental design

At the end of the initial study, monkeys were maintained in their home cage with ad libitum food intake and no exercise until they exhibited three normal menstrual cycles. Blood samples were collected daily during this time to determine whether animals displayed consistent peak plasma estradiol concentrations and peak luteal phase progesterone concentrations.

Results

Monkeys were categorized based on their reproductive hormone secretion and menstrual cyclicity during the two stress cycles. About one third of the monkeys continued to have menstrual cycles throughout the stress, retained normal cyclic patterns of ovarian steroid hormones, showed menses within 38 days for each stress cycle and continued to ovulate, as judged by the presence of an estradiol surge and progesterone secretion in the luteal phase (n=5; called high stress resilient, HSR; Fig. 2). About one third of the monkeys continued to show cyclic changes in estradiol and progesterone and menses during stress cycle 1 and showed menses within 38 days of initiating this cycle, but then showed a suppression of circulating estradiol and progesterone and failed to have a menstrual cycle in stress cycle 2 (n=6; called medium stress resilient, MSR; Fig. 3). The last one third of the monkeys showed an immediate suppression of circulating estradiol and progesterone concentrations and failed to have a menstrual cycle within 60 days of initiating stress exposure (n=4; called stress sensitive, SS; Fig. 4). Differences between groups for circulating hormone levels, length of the menstrual cycle, calorie intake, weight, and weight loss were assessed by a one-way analysis of variance, followed by a Student–Newman–Keuls post hoc test. A Bonferroni correction was used to account for multiple comparisons. Differences were considered significant for $p \leq 0.05$. All data are reported at mean±SEM.

Prior to stress exposure, there were significant differences between the SS and HSR groups in peak follicular phase estradiol levels and peak luteal phase progesterone levels (Fig. 5), with SS animals showing lower estradiol (SS 292±86 pg/ml; HSR 635±90 pg/ml, p=0.01) and progesterone (SS 15.2±4.8 ng/ml; HSR 31±4.5 ng/ml, p=0.001). There were no significant differences, however, between SS and HSR animals in circulating levels of LH or FSH either at the midcycle surge or during the rest of the cycle in control cycle 1. There were also no significant differences in length of the menstrual cycle, length of the

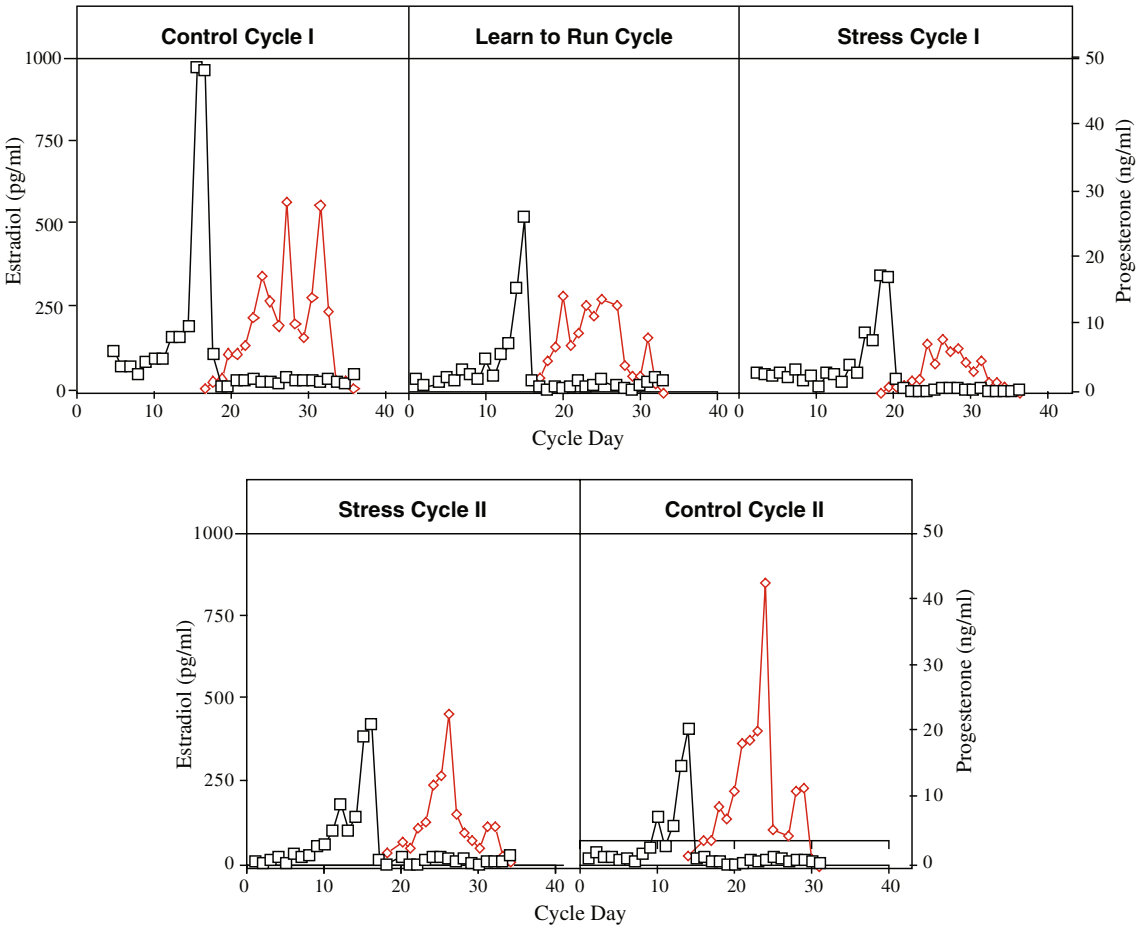

Fig. 2 Circulating levels of plasma estradiol (E; *open squares*) and progesterone (P; *open diamonds*) in a monkey which showed high stress resilience when exposed to combined psychosocial stress + diet + exercise. This monkey continued to display cyclic patterns of ovarian steroid hormones throughout stress exposure

follicular phase, and initial body weight or body weight loss between the groups (Table 1). In the second part of the study, significant differences remained throughout three control menstrual cycles in the peak serum estradiol and progesterone concentrations in the HSR and SS groups (Table 2).

Discussion

The results of this study show striking individual differences in the response of the reproductive axis to a moderate level of combined psychosocial and metabolic stress. About one third of the animals showed a rapid and profound suppression of reproductive hormone secretion (SS) while about one third retained normal menstrual cyclicity throughout 2 months of stress exposure (HSR). The final one third retained menstrual cyclicity during the initial phases of stress exposure but then lost cyclic secretion of reproductive hormones (MSR). Very interestingly, there was a significant difference in peak follicular phase estradiol concentrations and peak luteal phase progesterone concentrations between the HSR and SS groups, with the MSR group showing intermediate levels of these hormones, even before stress was initiated (in the control 1 cycle). The fact that this difference in peak ovarian steroid hormone levels was maintained across three consecutive control menstrual cycles suggests that secretion of ovarian steroid hormones is a stable characteristic of individuals with an increased propensity for sensitivity to stress-induced reproductive dysfunction. On the other hand, characteristics such as body weight, weight loss, menstrual cycle length, or length of the follicular phase appear to have no relationship to propensity to develop stress-induced reproductive dysfunction to a moderate, short-term stress exposure.

There would appear to be at least two possible mechanisms that could underlie the link between stress sensitivity and chronically lower circulating levels of ovarian steroid hormones. One possibility is that stress-sensitive individuals could have less GnRH and LH/FSH stimulation to the ovary resulting from either chronically

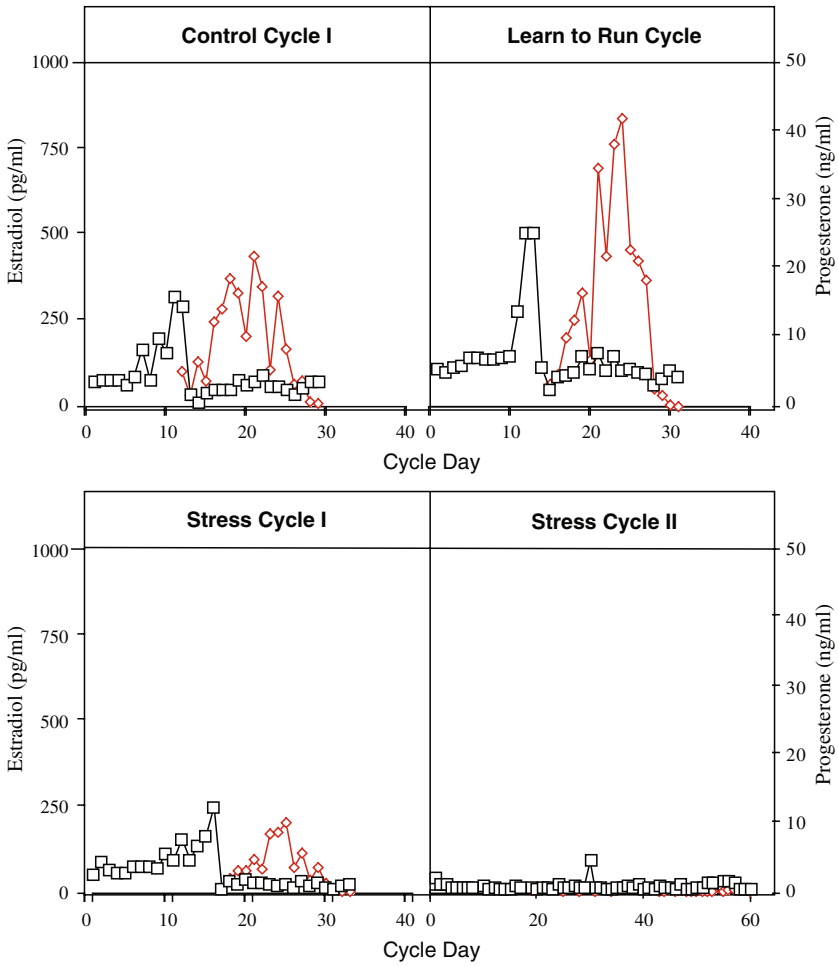

Fig. 3 Circulating levels of plasma estradiol (E; *open squares*) and progesterone (P; *open diamonds*) in a monkey which showed medium stress resilience when exposed to combined psychosocial stress + diet + exercise. This monkey continued to display cyclic patterns of ovarian steroid hormones when initially exposed to stress, but then became amenorrheic with further stress exposure

increased inhibitory input into the GnRH system or chronically decreased stimulatory input into GnRH neurons. In fact, a number of neural systems have been identified whose activity is altered by various forms of stress (e.g., corticotropin-releasing hormone, β-endorphin, neuropeptide Y, and norepinephrine) and each can in turn, alter the central neural drive to the reproductive axis [46]. However, our failure to detect significant differences between HSR and SS animals in serum LH or FSH concentrations across the cycle does not support this hypothesis. But, we note that in this study only a single daily blood sample was collected. Because gonadotropins are released in a pulsatile fashion it is possible that our failure to find differences in serum LH and FSH between HSR and SS groups may reflect the lack of sensitivity in our sampling method. A more sensitive method for examining differences in pulsatile gonadotropin secretion would be to chronically catheterize monkeys and collect frequent blood samples (at 10 min intervals) to accurately assess pulsatile LH secretion [35]. We are currently conducting this study in animals that have been adapted to a vest and tether for remote sampling.

Alternatively, another mechanism potentially linking differential stress sensitivity to varying levels of circulating ovarian steroid hormones is that low steroid hormone levels may lead to increased anxiety and sensitivity to stress. Estrogen and progesterone regulate many central neural systems that mediate anxiety. The following series of studies examined several pivotal neural systems involved in stress, anxiety, and reproductive function. Further studies are needed to determine if increased sensitivity to stress leads to lower ovarian steroid hormone secretion or whether lower ovarian steroid hormone secretion increases sensitivity to stress. It is also possible that there is a circular aspect of this relationship, such that individuals with a predisposition for stress sensitivity are more likely to have lower activity of the hypothalamic–pituitary–gonadal axis, which in turn heightens their sensitivity to stress by further decreasing ovarian steroid hormone levels in the brain.

We believe that it is likely that the results of this study, examining sensitivity to a complex psychosocial and metabolic stress, have direct relevance to a number of forms of stress-induced reproductive dysfunction. Forms of stress-induced reproductive dysfunction that are seen

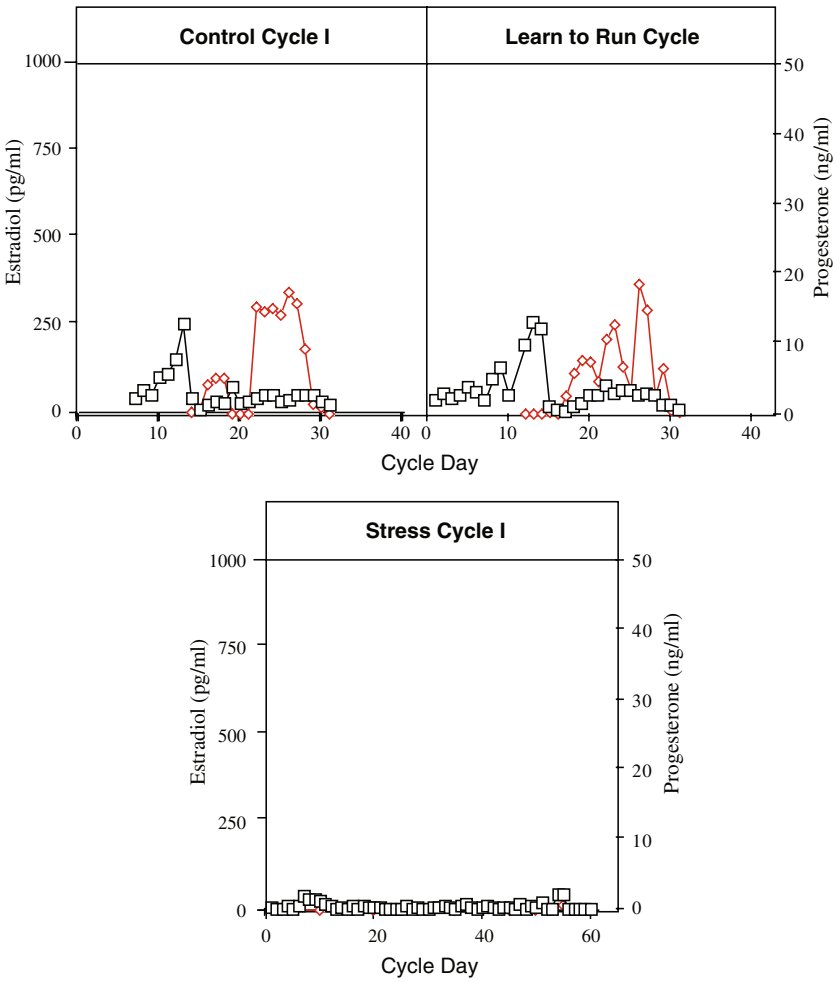

Fig. 4 Circulating levels of serum estradiol (E; *open squares*) and progesterone (P; *open diamonds*) in a monkey which showed stress sensitivity when exposed to combined psychosocial stress + diet + exercise. This monkey immediately became amenorrheic upon stress exposure

clinically include functional hypothalamic amenorrhea, anorexia and bulimia nervosa, and exercise-associated amenorrhea. Although it was initially believed that each of these syndromes represented a fairly discrete type of stress (i.e., functional hypothalamic amenorrhea: a psychosocial stress; anorexia nervosa: a nutritional stress; exercise-associated amenorrhea: an exercise stress), there is a growing recognition that each of these syndromes involves exposure to a combination of stresses. As discussed in the "Introduction", there is strong evidence that functional

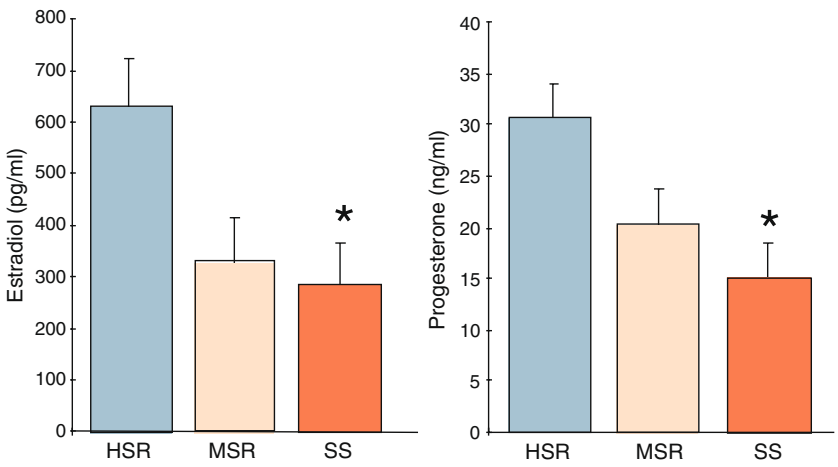

Fig. 5 Peak follicular phase plasma estradiol (E) concentrations (*left panel*) and peak luteal phase plasma progesterone (P) concentrations (*right panel*) in HSR, MSR, and SS monkeys during the control 1 menstrual cycle. *Asterisks* indicate a significant difference between groups, $p<0.05$

Table 1 Menstrual cycle and weight parameters by stress-sensitivity category

Stress-sensitivity category	Menstrual cycle length (days)	Follicular phase length (days)	Weight (kg)	Weight change
HSR	31.5±1.9	16.2±2.3	4.1±0.3	0.34±0.6
MSR	33.9±2.3	16.8±3.6	3.9±0.4	0.3±0.8
SS	30.7±1.6	15.7±4.1	4.2±0.4	0.32±0.7

hypothalamic amenorrhea involves both psychosocial and metabolic stresses. Reproductive dysfunction occurring in patients with anorexia nervosa was considered primarily the result of severe nutritional stress; however, it is well documented that a substantial percentage of anorexic patients do not experience normalization of reproductive function with weight restoration [47, 48]. The propensity to exercise intensively [49] and the significant psychological stress associated with weight gain in anorexic patients [50] are likely contributors to the long-term suppression of the reproductive axis. Likewise, in bulimia nervosa there is disordered eating, but this is often accompanied by increased exercising, and these patients experience significant psychological stress [51]. Recent studies of exercise-associated amenorrhea indicate that within clinical populations that show reproductive dysfunction there is often simultaneous calorie restriction [52–54]. Thus, clinically relevant forms of stress-induced reproductive dysfunction all involve simultaneous exposure to multiple forms of stress.

In summary, this model established that there are individual differences in sensitivity of the reproductive axis to stress-induced impairment, even for a relatively moderate form of stress. Prior activity of the reproductive axis, in a non-stressed condition, can be used to identify individuals that are stress sensitive versus stress resilient, in that peak secretion of estradiol and progesterone over a menstrual cycle appears to be a fairly stable individual characteristic. This finding suggests that the development of strategies for recognizing stress-sensitive individuals and then providing targeted therapy to increase stress resilience may be possible in the future.

The next series of studies questioned whether there are endogenous underlying differences in central neural systems between stress-resilient and stress-sensitive monkeys which could account for the differences in steroid hormone secretion and the differences in their ability to maintain ovulation in the presence of stress. One of the most important neural systems governing an animal's state of arousal and ability to cope with stress is the serotonin neural system [55].

Global Differences in the Serotonin Neural System

Introduction

The serotonin neural system plays a pivotal role in mood and affective regulation, cognition, satiety, and in numerous autonomic functions [56–58]. Decreased activity of the central serotonin system is found in individuals with increased stress sensitivity and anxiety disorders [59–61]. In addition, stress impacts serotonin function in a variety of ways depending on the intensity and duration of the stress [62–64]. Although many studies have shown individual responses to stress, we probed the neural function of animals long after the stress was removed and the animals were all cycling normally. We hypothesized that monkeys that show sensitivity of the reproductive axis to stress may have lower activity of the central serotonin system.

Thus, the next goal was to determine whether there are differences in endogenous function of the central serotonergic system in stress-sensitive versus stress-resistant animals *in the absence of stress*. We used fenfluramine administration followed by measurement of prolactin and cortisol to access global serotonin availability [65, 66]. Fenfluramine causes an immediate release of serotonin via transporter reversal and blocks serotonin reuptake thereby causing a rapid elevation of extracellular serotonin. Fenfluramine-induced release of pituitary hormones thus reflects the level of endogenous activity in the serotonergic neurons regulating hypothalamic neuroendocrine systems [67].

Methods

Thirteen female cynomolgus macaques described above were used. Two animals were lost due to clinical reasons.

Table 2 Peak estradiol and progesterone concentrations in HSR and SS animals across three control menstrual cycles

Stress-sensitivity category	Cycle 1 E2 (pg/ml)	Cycle 2 E2 (pg/ml)	Cycle 3 E2 (pg/ml)	Cycle 1 P4 (ng/ml)	Cycle 2 P4 (ng/ml)	Cycle 3 P4 (ng/ml)
HSR	734±91*	603±98*	622±54*	25±3.8*	23±2.4*	31±5.6*
SS	452±56	431±59	367±92	14±2.3	9.8±4.1	13.6±3.3

*$p \leq 0.05$ indicates a significant difference, compared to SS animals in the same cycle

The animals were housed in single cages and monitored daily for menstruation. Upon detection of menstruation, the animals were scheduled for fenfluramine challenge before day 5 of the follicular phase of their cycle in July 2001 and for thyrotropin-releasing hormone (TRH) and corticotrophin-releasing hormone (CRF) challenge in September 2001.

On day 1 of a non-stressed menstrual cycle, animals were scheduled for a fenfluramine challenge before day 5 of the cycle. On the day of the challenge, each animal was sedated with ketamine (100 mg) in their home cage and transported to a surgical suite. The monkeys were placed on a temperature-regulated surgical table and connected to vital sign monitors under the supervision of the veterinary surgical staff. Fenfluramine challenges and control challenges of TRH + CRH were administered under propofol anesthesia as previously published [68].

A two-factor ANOVA was conducted on the data with group and time as the dependent variables using the Statistix Analytical Software package (Tallahassee, FL, USA). Specific post hoc comparisons were made by Tukey's analysis with a Bonferroni correction for multiple comparisons. The treatment response within a group was analyzed with a non-parametric ANOVA (Freidman's) for repeated measures followed by Dunn's post hoc comparison. When indicated, comparisons were made with Student's or Welch's *t* test as determined by variances. Differences were considered significant if $p<0.05$. Data is presented as mean±SEM.

Results

As shown in Fig. 6, top panel, prolactin secretion was significantly different between the experimental groups (two-way ANOVA, $p<0.0001$). Prolactin levels before fenfluramine challenge were higher in the stress-sensitive group than in the high- or medium-stress-resistant groups ($p<0.01$). However, stress-sensitive animals had a lower response to fenfluramine compared to high-stress-resistant animals (post hoc test HSR versus SS, $p<0.001$). The prolactin response of the medium-stress-resistant animals did not differ from the other two groups. Cortisol secretion in response to fenfluramine (Fig. 6, bottom panel) was also significantly different between the experimental groups (two-way ANOVA, $p<0.0001$; post hoc test HSR versus SS, $p<0.001$). Stress-sensitive animals had a greater release of cortisol compared to high stress-resilient animals, with the medium-stress-resistant animals again showing no significant difference from the other two groups. In contrast, prolactin secretion in response to the thyrotropin-releasing hormone challenge was not suppressed in the stress-sensitive group, and the response to CRH challenge was similar between the groups [68]. Thus, a difference in the pituitary stores of prolactin or ACTH cannot explain the results.

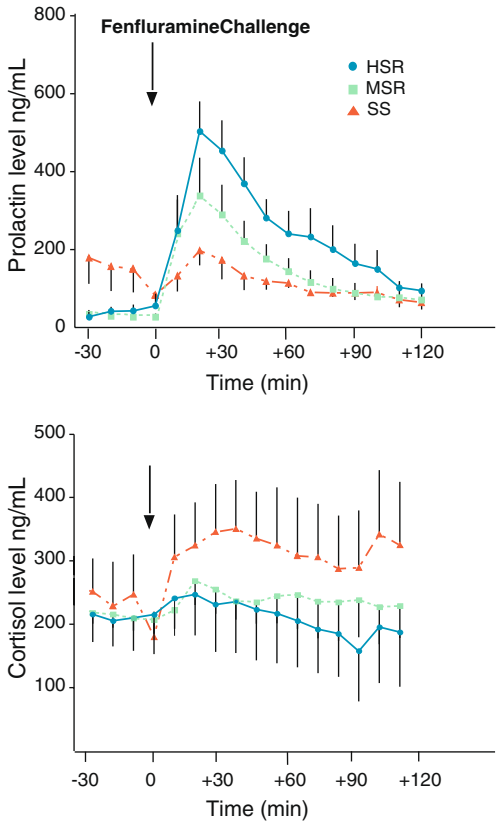

Fig. 6 Prolactin (*top*) and cortisol (*bottom*) responses to an injection of fenfluramine (5 mg/kg, i.v.) in saline while monkeys were maintained under propofol anesthesia. In the *top panel*, there was a significant difference between the groups in the amount of prolactin secreted after fenfluramine (two-way ANOVA, $p<0.0001$), with the HSR group secreting significantly more prolactin compared to the SS group (Tukey's post hoc test, $p<0.001$). In the *bottom panel*, the cortisol response to fenfluramine was significantly different between the experimental groups (two-way ANOVA, $p<0.0001$), with the SS group secreting more cortisol compared to the HSR group (Tukey's post hoc test, $p<0.001$). Reprinted from Bethea et al. [68]

Discussion

Serotonin appears to be a key neurotransmitter in the regulation of mood, including affective state and anxiety levels [55]. Moreover, it has been generally hypothesized that a diminished capacity of the serotonergic system may underlie a heightened susceptibility to stress as well as vulnerability to depression and/or drug abuse [69, 70]. Decreases in ovarian steroid hormones also lead to decreases in various aspects of serotonin neural function [71]. These data support the notion that the serotonin system of stress-sensitive individuals has a lower functional capacity than that of stress-resilient individuals even in the absence of stress, and it supports the long-standing hypothesis that individuals with heightened sensitivity to stress have diminished serotonin function.

Nonetheless, the stress-sensitive group had a higher release of cortisol than the stress-resilient group. This is confounding if cortisol release also reflects the serotonin capacity. There are two potential explanations. One possibility is that in the stress-sensitive individuals, the CRH neurons driving the hypothalamic–adrenal axis are super-sensitive to serotonin. Earlier work demonstrated that serotonergic denervation increases the functional neuroendocrine response of the hypothalamic–pituitary–adrenal (HPA) axis to serotonin agonists [72]. Hence, in the stress-sensitive group, even the lower amount of serotonin that was released by fenfluramine may have acted upon super-sensitive CRF neurons. This line of reasoning is supported by a report that different pathways from the mediobasal hypothalamus mediate serotonergic stimulation of prolactin and corticosterone secretion in rodents [73, 74]. The cellular or molecular mechanism involved in the switch of axis sensitivity between stress-sensitive and stress-resilient animals could be of great interest. As described below, we found that indeed, CRH is higher in stress-sensitive individuals in the absence of stress.

Rodent studies also suggest that the effect of fenfluramine on corticosterone is not mediated by serotonin release [75]. If this is true in primates, then perhaps the non-serotonin-mediated effect of fenfluramine on cortisol is greater in stress-sensitive than in stress-resilient animals. However, the nature of the non-serotonin mechanism remains unresolved. In a similar manner, fenfluramine challenges in alcoholic patients produce lower prolactin and higher cortisol secretion than in non-alcoholic controls [76, 77].

Exactly how stressors are transduced by the brain into perceptions of stress, physiological stress responses, and deleterious effects on mood and many other health outcomes is not understood. However, this study probed neural function in non-stressed animals that had a previously documented difference in reproductive function under stress, and we show that there are endogenous differences in serotonin capacity even in the non-stressed state. It is attractive to speculate that the lower endogenous serotonin makes the individual more sensitive to stress. Thus, the "stressfulness" of a stimulus may reside more in the individual nervous system than in the stimulus. Our animals were individually housed and not stressed at the time of the fenfluramine challenge, so the basal cortisol secretion was not a variable between the groups. Thus, the differences observed in stress sensitivity may have resulted from differences in genetic predisposition or early rearing experiences, factors known to influence activity of the hypothalamic–pituitary–adrenal axis [78]. A relationship between low socioeconomic status and low serotonergic activity, also measured by the prolactin response to fenfluramine, has been observed in a study of men and women [79].

From the earlier study in which reproductive function was characterized, we know that the stress-sensitive animals have lower peak and lower average estradiol and progesterone levels across three non-stressed menstrual cycles. Indeed, ovarian steroid production appears to be a stable endogenous and individual state. QTL studies in baboons have found a microsatellite polymorphism that has a significant effect on estrogen [80]. The corpus luteum is the major source of progesterone and the health and function of the corpus luteum depends largely on the viability of the ovulatory follicle [81, 82]. Thus, it is not surprising that levels of estrogen and progesterone during a non-stressed menstrual cycle correlated with each other, and that both reflect the magnitude of sensitivity of the reproductive axis to stress.

In summary, the stress sensitivity of cynomolgus macaques correlates with prolactin secretion in response to the serotonin releaser, fenfluramine, suggesting that highly stress-resistant animals have higher levels of endogenous serotonin than stress-sensitive animals even in the absence of stress. In the next study, we examined gene expression related to serotonin neural function in stress-sensitive versus stress-resilient individuals.

Serotonin Gene Expression

Introduction

Serotonin neurotransmission is generally thought of as a combination of synthesis, release, turnover, neural activity, and degradation. Pivotal proteins governing these functions are translated from messenger RNAs (mRNAs) coding tryptophan hydroxylase 2 (TPH2), the serotonin reuptake transporter (SERT), the 5HT1A autoreceptor, and the monoamine oxidases A and B (MAO-A, MAO-B).

Therefore, we questioned whether the difference in the functional capacity of the serotonin system could be due to differences in the expression of four genes (SERT, 5HT1A, MAO-A, MAO-B) within serotonin neurons of the dorsal raphe nucleus or due to differences in serotonin cell number. We also questioned the expression of tryptophan hydroxylase (TPH), but at the time only TPH1 was known and the gene for TPH2 had not been discovered [83, 84]. When TPH2 was discovered, the sections of the dorsal raphe from these animals were depleted.

Methods

After completion of all in vivo protocols, the animals were monitored daily for menstruation. Upon detection of menstruation, the animals were scheduled for euthanasia

before day 5 of the follicular phase of their cycle during November/December 2001.

The monkeys were euthanized according to the procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta. A blood sample was obtained at necropsy for determination of estrogen and progesterone concentrations at the time of death. The brain was perfused and 25 μm sections through the midbrain raphe region were obtained as previously described [85]. Reverse transcriptase-polymerase chain reaction (RT-PCR) for a polymorphism in the serotonin reuptake transporter promoter region (5HTTLPR) was conducted on genomic DNA extracted from whole blood as previously described [85].

In situ hybridization for SERT, 5HT1A, MAO-A, and MAO-B; densitometric analysis of the autoradiograms; and analysis of the data were previously published [85]. Six anatomical levels of the dorsal raphe nucleus were examined in a rostral to caudal direction at 250 μm intervals. All statistical analyses were conducted using the Prism Statistic Program (GraphPad, San Diego, CA, USA). A confidence level of $p<0.05$ was considered significant.

Results

Specific signals for SERT, 5HT1A autoreceptor, MAO-A and MAO-B mRNAs were detected in the dorsal raphe and representative photomicrographs of the autoradiographic signals for each transcript are shown in [85].

SERT

Examination of SERT across six levels of the dorsal raphe indicated that there was a significant decrease in expression in the caudal levels. Hence, the overall mean optical density (OD) was calculated and then, mean was obtained for the rostral four levels and the caudal two levels. As shown in Fig. 7, top panel, the stress-sensitive group exhibited a significant decrease in the SERT OD in the caudal levels of the dorsal raphe, but not in the rostral levels. In the overall mean of all levels, SERT expression tended to decrease in the MSR and SS groups, but it did not reach statistical significance. Analysis of positive pixel area yielded similar results. SERT OD in the dorsal raphe was also significantly correlated with serum P concentrations measured during the pre-stress control menstrual cycle, i.e., higher SERT mRNA signal was observed in animals with higher serum P concentrations (Fig. 7, bottom panel). These animals also had the highest degree of stress resilience.

5HT1A

There was no difference between the groups in 5HT1A mRNA. Nonetheless, the 5HT1A OD in the entire raphe was highly correlated with serum P concentrations measured during the pre-stress control menstrual cycle wherein the animals with the greatest 5HT1A OD exhibited the highest serum P concentrations [$r^2=0.5917$; $p=0.009$].

MAO-A

MAO-A mRNA, which codes for the serotonin degradative enzyme, is a low abundance mRNA in the dorsal raphe. However, MAO-A mRNA decreased significantly across all six levels of the dorsal raphe as stress resilience decreased. There was markedly less overall MAO-A OD in stress-sensitive and medium-stress-resilient animals than in highly stress-resilient animals (Fig. 8, top panel). The positive pixel area for MAO-A signal also decreased significantly in the stress-sensitive animals. MAO-A OD was also significantly correlated with serum P concentrations during the pre-stress control menstrual cycle with the highest expression of MAO-A observed in the animals with the highest serum P concentrations (Fig. 8, bottom panel).

MAO-B

MAO-B mRNA codes for the enzyme that preferentially degrades catecholamines and it is expressed abundantly in the dorsal raphe. MAO-B mRNA exhibited a decrease in both OD and pixel areas in stress-sensitive groups, in a manner similar to MAO-A, but the differences were not statistically significant. However, MAO-B OD signal was also highly correlated with serum P concentrations during the pre-stress control menstrual cycle, with the highest expression of MAO-B occurring in animals with the highest peak of serum P during a normal control cycle [$r^2=0.36733$; $p=0.0036$].

Serotonin and Genomics

Three levels of the dorsal raphe were chosen for antigen retrieval, serotonin immunocytochemistry, and counting as previously described [85]. After combining the cell number for all levels for each animal and then obtaining the mean for each group, there was a significant difference across the groups (ANOVA $p=0.0243$). The SS group had significantly fewer cells than the HSR group (p, 0.05, Tukey's), and the MSR group was in between the HSR and SS group (Fig. 9).

RT-PCR analysis of the polymorphic locus 2 in the promoter region of the serotonin reuptake transporter gene, 5HTTLPR, indicated that all of the monkeys in this study contained only the long allele of this polymorphism.

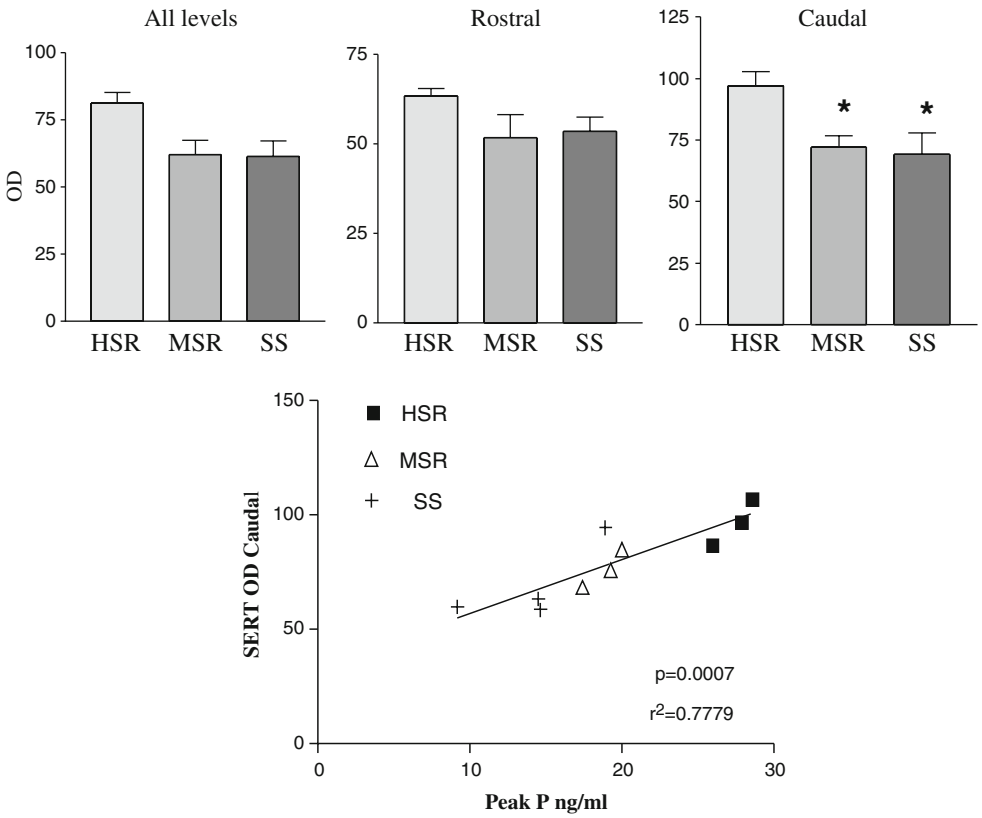

Fig. 7 *Top panel, far left*. The mean optical density for SERT signal for all six levels of the DRN was obtained for each animal. Then the mean of the animals in each group was obtained so the SEM represents the variance between animals. There was no significant difference in the mean OD (all levels 1–6) for SERT between the groups. Then, the DRN was parsed into rostral (levels 1–3) and caudal (levels 4–6) segments and the mean OD was obtained (*center* and *right panels*). The SEM again represents the variance between animals. In this analysis, there was a significant decrease in caudal DRN SERT expression in the medium-stress-resistant and stress-sensitive groups compared to the high-stress-resistant group ($p<0.05$, ANOVA followed by Tukey's). *Bottom panel*. A regression analysis was performed with the mean SERT OD of each individual animal (average across six levels) versus the peak serum progesterone concentration of the same animal obtained 3 years earlier during a control, non-stressed menstrual cycle. There was a significant positive correlation between SERT mRNA OD and peak progesterone levels from a non-stressed cycle ($p=0.0007$). Reprinted from Bethea et al. [85]

Subsequent reports indicate that cynomolgus macaques are not polymorphic at this locus.

Discussion

We followed up our initial fenfluramine responsiveness findings by assessing the level of expression for four genes in serotonin neurons and then counting the number of serotonin-positive neurons at three levels of the DRN. We found that overall expression for this group of genes in the dorsal raphe is compromised to a lesser or greater extent in stress-sensitive monkeys, even in the absence of stress, and that the decrease in gene expression may reflect the decrease in serotonin cell number in stress-sensitive animals. This study reinforces the notion that serotonin neural function is compromised in stress-sensitive individuals and shows, for the first time in primates, that there are fewer serotonin neurons and less overall gene expression, in stress-sensitive individuals. Two mRNAs pivotal for serotonin function (SERT and MAO-A) are significantly lower in stress-sensitive compared to stress-resilient animals. Two other mRNAs that play a role in serotonin function (5HT1A and MAO-B) show a decremental trend in the stress-sensitive animals compared to the stress-resilient animals. More importantly, all four of these mRNAs show a significant correlation with serum levels of progesterone during a non-stressed control cycle, which were significantly lower in stress-sensitive animals. None of the variables examined had any correlation to the serum concentrations of E or P at the time of euthanasia. We have no method for integrating changes across different genes, but it seems clear that gene expression in the serotonin system is compromised in stress-sensitive animals in the absence of stress. Moreover, analysis of these data as a population continuum is more reflective of physiology than dividing them into discrete groups.

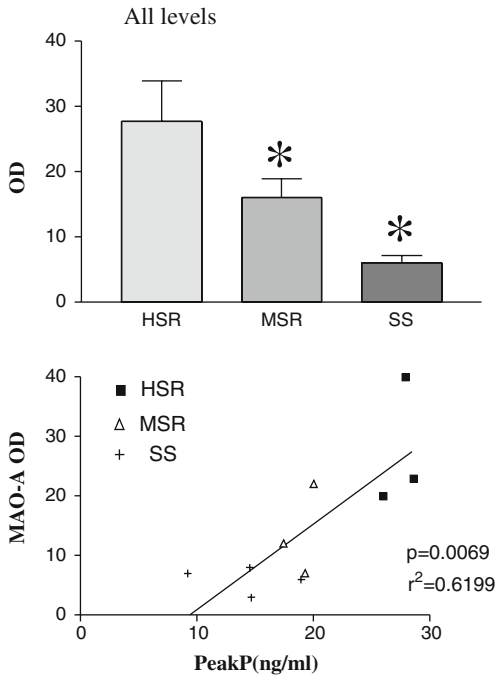

Fig. 8 *Top panel*. The mean optical density for MAO-A mRNA signal for all six levels of the DRN was obtained for each animal. Then, the mean of the animals in each group was obtained so the SEM represents the variance between animals. There was a significant decrease in MAO-A signal in the medium-stress-resistant and stress-sensitive groups compared to the high-stress-resistant group ($p<0.007$, ANOVA followed by Tukey's). *Bottom panel*. A regression analysis was performed with the mean OD for MAO-A of each individual animal (average across six levels) versus the peak serum progesterone concentration of the same animal obtained 3 years earlier during a control, non-stressed menstrual cycle. There was a significant positive correlation between MAO-A mRNA OD and peak progesterone levels from a non-stressed cycle ($p=0.0069$). Reprinted from Bethea et al. [85]

Subsequent to the conduct of these experiments, a second isoform of TPH was discovered called TPH2 that is responsible for the synthesis of brain serotonin. We examined sections from our animals for TPH mRNA, but at the time only TPH1 was known [86]. Of the 251 bp in our TPH1 complementary DNA (cDNA) a 230-bp stretch is 65% homologous to TPH2. We cannot rule out the possibility that it was hybridizing to TPH2 under the stringency conditions employed. Due to this uncertainty, we cannot report the results with confidence and the raphe sections from these animals are depleted. Therefore, TPH2 was recently examined in new groups of animals. TPH2 was significantly lower in stress-sensitive animals than in highly stress-resilient animals (unpublished and data not shown).

There is a body of literature showing that stress can lead to secondary disease including reproductive dysfunction in sensitive individuals [3, 29, 46]. Decreased activity of central serotonin pathways is linked to a number of stress-sensitive psychiatric disorders including anxiety and depression [61, 87–89], bipolar disorder [90], alcoholism [77], anorexia nervosa [91], and premenstrual syndrome [92]. Both stress and circulating levels of ovarian steroid hormones can influence serotonin neurotransmission [71, 93]. The serotonin neural system also contributes to aspects of cognition, learning, and memory [94, 95, 96]. Hence, it has become an important site for pharmacologic intervention in psychiatric disorders and increasingly in stress-related physiological disorders. Moreover, the same risk factors that lead to an increased incidence of anxiety disorders also lead to an increased incidence of other stress-related problems including growth retardation [97], type 2 diabetes [98], and rheumatoid arthritis [99]. Sensitivity to stress-induced reproductive dysfunction may fall in this same category.

The observation that stress-sensitive animals have fewer serotonin neurons was unexpected. We previously reported that there were no differences in serotonin cell number in spayed rhesus macaques with or without 1 month of hormone therapy (HT) [100], and human studies have not observed differences in serotonin cell number in depressed suicides versus controls [101]. However, other studies found fewer DRN neurons in diseases such as Alzheimer's [102] and alcoholism [103]. A long-term experiment is underway in our Japanese macaque troop to test the hypothesis that ovarian hormones protect serotonin neurons from apoptosis.

The data from the stress-sensitive animals do *not* directly reflect the earlier studies with hormone replacement [71,

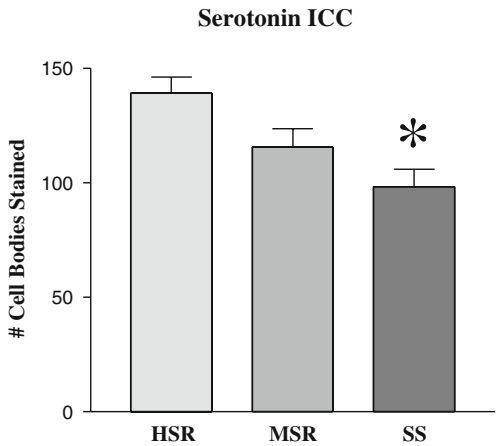

Fig. 9 The number of serotonin-positive neurons on the right side of the dorsal raphe nucleus was obtained at three anatomical levels. The mean of all of the levels was obtained for each animal and then the overall mean was obtained for the group so that the SEM represents the variance between animals. There were significantly fewer serotonin neurons in the SS group (ANOVA, $p<0.03$). *Significantly different from HSR with Tukey's post hoc pairwise comparison, $p<0.05$. Reprinted from Bethea et al. [85]

104] because of the differences in serotonin neuron number. We previously found that estrogen (with or without progesterone) treatment of spayed rhesus macaques decreases SERT, 5HT1A, and MAO-A mRNA expression in the DRN. In this study, we observed the highest expression of SERT, 5HT1A, and MAO-A on autoradiograms in the animals with the greatest number of serotonin neurons and highest serum progesterone levels. We found lower gene expression signals in animals with fewer neurons. Thus, although estrogen and progesterone regulate serotonin gene expression, they are not directly or wholly responsible for the changes in gene expression in the different stress-sensitive groups. Rather, we believe that the lifetime secretion of ovarian steroids may be preventing cell death or altering the set point of function in the serotonin system.

We speculate that when animals of similar temperament and serotonin cell number, like the spayed rhesus monkeys, are acutely treated with ovarian hormones, then the mRNA regulation previously observed will be manifested, that is downregulation of SERT, 5HT1A, and MAO-A. In this study, the animals were all euthanized in the early follicular phase of a non-stressed menstrual cycle when estrogen and progesterone levels were similar across the groups. Retrospectively, the animals with the highest cyclic secretion of progesterone had the most serotonin neurons, and they yielded higher gene expression signals on autoradiograms, i.e., elevated SERT, 5HT1A, and MAO-A compared to stress-sensitive animals. Hence, in this model, the gene expression detected on the films may be a consequence of the serotonin cell number or viability and not acute hormone levels.

Neurodegenerative diseases have become a major target for the development of pharmacotherapies. These diseases share synaptic loss, neuronal atrophy, and death as common pathological hallmarks. Recent data suggest that depression, mood disorders, and other mental illnesses may have a degenerative component and that antidepressants contribute to neural plasticity and cellular resilience [105, 106]. Estrogen has been shown to be neuroprotective in other brain regions [107, 108], and so it is possible that ovarian hormones are neuroprotective for serotonin neurons as well. Therefore, we hypothesize that animals with higher lifetime secretion of ovarian steroids will experience decreased serotonin neuronal death and greater cellular resilience. The maintenance of a larger population of serotonin neurons will promote stress resilience due to greater overall serotonin availability and neurotransmission. It is attractive to speculate further that the lower serotonin cell number and gene expression makes the individual more sensitive to stress and stress-induced reproductive dysfunction.

In summary, the number of serotonin neurons and overall expression of pivotal genes in serotonin neurons correlates strongly with the stress sensitivity of the individual and with the peak of progesterone secretion during non-stressed control cycles. That is, animals with high stress resistance have the highest lifetime secretion of progesterone, the greatest number of serotonin neurons, and the highest expression of serotonin-related genes in the dorsal raphe nucleus. These strong associations between progesterone, degree of stress sensitivity, and serotonin warrant further investigation to determine causal relationships within these systems.

Next, we questioned whether serotonin receptors and other systems that impact GnRH release are altered in stress-sensitive individuals. Therefore, we began our exploration of the hypothalamic systems that impact GnRH release and the control of the reproductive axis. First, we examined the serotonin receptors, and then we examined the expression of GAD67, the enzyme responsible for the synthesis of the major inhibitory neurotransmitter, GABA. Subsequently, we examined CRH and pro-opiomelanocortin (POMC) gene expression as two additional systems believed to inhibit GnRH.

Hypothalamic Systems: Serotonin Receptors and GABA

Introduction

The hypothalamus receives abundant innervation from the raphe serotonergic system [109, 110] and expresses serotonin 5HT1A, 2A and 2C receptors [111, 112]. Hypothalamic serotonin is involved in the regulation of GnRH secretion through systems that regulate GnRH neurons and that are influenced by stress. Among them, gamma-aminobutyric acid provides the major input to GnRH neurons [113–115]. There is evidence that serotonin inhibits LH secretion in rats through the stimulation of GABA neurons [116]. In the monkey, GAD67 (the rate-limiting enzyme in the synthesis of GABA) expressing neurons localized in the infundibulum co-express the serotonin 5HT2C receptor. Estrogen treatment suppressed both GAD67 [117] and 5HT2C receptor [112] gene expression, suggesting the participation of these neurons in the control of ovulation in monkeys.

We hypothesized that the lower serotonergic input from the dorsal raphe nucleus to the hypothalamus, or the lower concentrations of estrogen and progesterone in the stress-sensitive animals may affect the expression of 5HT receptors in the hypothalamus, as well as the expression of GAD67 in GABA neurons that are regulated by serotonin. The same animals from which the midbrain raphe was obtained also provided the hypothalami for the following studies.

Methods

In situ hybridization for 5HT1A, 5HT2A and 5HT2C receptors, and GAD67, densitometric analysis of the autoradiograms and analysis of the data was previously published [118]. Prehybridization, hybridization, and wash temperatures were empirically optimized for each probe. The development, sequence, and characterization of the monkey specific 5HT1A, 5HT2A, and 5HT2C receptors, and GAD67 cDNAs and riboprobe hybridization were published previously [112, 117, 119]. All statistical analyses were conducted using the Prism Statistic Program (GraphPad, San Diego, CA, USA). A confidence level of $p<0.05$ was considered significant.

Results

5HT1A mRNA Expression in the VMN of HSR, MSR, and SS Monkeys

In macaque hypothalamus, the ventromedial nucleus (VMN) exhibited the densest expression of 5HT1A receptor mRNA [112] and also expressed estrogen and progestin receptor mRNA [120]. Therefore, four levels of the VMN were examined for 5HT1A mRNA expression in the characterized groups. There were no differences between groups in either 5HT1A pixel area or optical density at any level of the VMN (data published in [118]).

5HT2A mRNA Expression in the PVN of HSR, MSR, and SS Monkeys

We showed that 5HT2A receptor mRNA expression was most prominent in the hypothalamic paraventricular nucleus (PVN) and mammillary nuclei [112]. Since the PVN plays a pivotal role in neuroendocrine regulation, we examined 5HT2A mRNA expression in five levels of the PVN of the characterized groups. Figure 10, left panel, shows the overall mean positive pixel area and OD of 5HT2A ISH signal in the PVN. The average 5HT2A pixel area and OD were significantly higher in the SS animals ($p<0.05$).

5HT2C mRNA Expression in the Infundibulum of HSR, MSR, and SS Monkeys

We showed that the 5HT2C receptor mRNA was densely expressed and regulated by ovarian steroid hormone replacement in the infundibular region of macaques [112]. This region contains dense populations of estrogen and progestin receptor expressing neurons as well [120], and it plays a crucial role in the regulation of reproductive function. Therefore, we examined 5HT2C mRNA expression in three levels of the infundibular nucleus of the characterized groups. Figure 10, middle panel, shows that SS animals exhibited significantly higher 5HT2C-positive pixel area and OD in the overall average of all levels of the infundibulum, compared with HSR animals ($p<0.05$; Kruskal–Wallis ANOVA, followed by Dunn's post hoc test).

GAD67 mRNA Expression in the Infundibulum of HSR, MSR, and SS Monkeys

We previously showed that GAD67 mRNA expression was also robust in the macaque infundibulum and infundibular GABA neurons expressed 5HT2C mRNA [117]. Moreover, the infundibular nucleus contains dense populations of estrogen and progestin receptor containing neurons and GAD67 was suppressed by ovarian steroid hormone replacement. Therefore, we examined GAD67 mRNA expression at three levels of the infundibular nucleus of the characterized groups. As illustrated in Fig. 10, right panel, SS monkeys exhibited significantly higher average GAD67-positive pixel area and OD, compared with HSR individuals ($p<0.05$; Kruskal–Wallis ANOVA followed by Dunn's post hoc test). There was no significant difference in the levels of GAD67 mRNA in the infundibulum of MSR monkeys, compared with HSR animals. The same pattern of expression was observed in the posterior hypothalamus (PH) [118]. In addition, infundibular GAD67 mRNA was positively correlated with 5HT2C mRNA ($R=0.644$, $p=0.019$), and negatively correlated with luteal phase serum progesterone ($R=0.732$, $p=0.016$).

Discussion

These experiments show that stress-sensitive monkeys exhibited higher levels of 5HT2A receptor mRNA in the PVN, higher levels of 5HT2C receptor mRNA in the hypothalamic infundibular region, as well as higher levels of GAD67 mRNA in the infundibulum and the PH, compared with stress-resilient individuals, but there was no change in the expression of 5HT1A receptor mRNA levels in the VMN.

It is notable that the expression of 5HT1A receptor in the hypothalamic VMN is not different between stress-sensitive, medium stress-resilient, and highly stress-resilient monkeys. The dense expression of 5HT1A in the VMN is consistent with our previous observations [112]. However, 5HT1A mRNA in the VMN was not regulated by ovarian steroid hormone replacement, in spite of a robust population of steroid hormone receptor expressing neurons. Therefore, neither the difference in ovarian hormone concentrations nor the predicted difference in serotonin production between stress-sensitive and

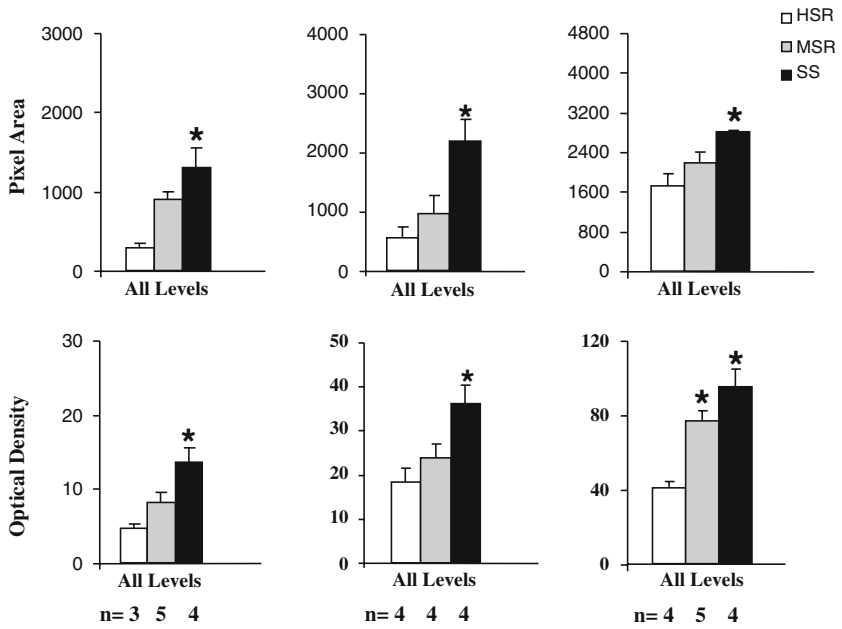

Fig. 10 5HT2A, 5HT2C, and GAD67 mRNA expression in hypothalamus of HSR, MSR, and SS monkeys. *Left panel*. 5HT2A optical density (OD) and pixel area were obtained at five levels of the PVN of HSR, MSR, and SS monkeys. The mean of all of the levels was obtained for each animal and then the overall mean was obtained for the group so that the SEM represents the variance between animals. There was significantly more 5HT2A mRNA in the PVN of SS animals compared to HSR animals. *Middle panel*. 5HT2C optical density (OD) and pixel area were obtained at three levels of the infundibulum of HSR, MSR, and SS monkeys. The mean of all of the levels was obtained for each animal and then the overall mean was obtained for the group so that the SEM represents the variance between animals. There was significantly more 5HT2C mRNA in the infundibulum of SS animals compared to HSR animals. *Right panel*. GAD67 optical density (OD) and pixel area were obtained at three levels of the infundibulum of HSR, MSR, and SS monkeys. The mean of all of the levels was obtained for each animal and then the overall mean was obtained for the group so that the SEM represents the variance between animals. There was significantly more GAD67 mRNA in the infundibulum of SS animals compared to HSR animals. *$p<0.05$, Kruskal–Wallis one-way ANOVA, Dunn's post hoc test compared with HSR animals. The number of animals used in each group is shown below the histograms. Reprinted from Centeno et al. [118]

stress-resilient animals impacted the expression of the 5HT1A receptor in the VMN.

The serotonin 5HT1A receptor is coupled to protein Gi, reducing adenylyl cyclase and/or increasing the opening of K^+ channels [121, 122]. The hypothalamic 5HT1A receptor is involved in the inhibitory action of serotonin on female rodent sexual behavior. Administration of 5HT1A agonists into the VMN inhibits lordosis behavior in rats [123, 124]. In addition, serotonin in the VMN plays a modulatory role in the regulation of feeding behavior [125, 126]. The VMN contains few if any neuroendocrine neurons (neurons that project to the median eminence and regulate anterior pituitary hormone secretion). Our indicator of stress sensitivity is ovulation, which is controlled by neuroendocrine areas outside of the VMN. Along this line of reasoning, serotonin circuits that are not involved in mediating stress to neuroendocrine neurons may not be different between the groups.

The dense concentration of 5HT2A mRNA in the PVN is in agreement with our previous observations in pigtail macaques [112]. In this study, stress-sensitive monkeys exhibited higher levels of 5HT2A mRNA in this nucleus, compared with stress-resilient individuals. In contrast, ovarian steroid hormone replacement had no effect on 5HT2A expression [112]. Thus, it is attractive to speculate that the decrease in the activity of the serotonin system in stress-sensitive animals, and not the difference in ovarian hormone secretion, led to an upregulation of the 5HT2A receptor in the stress-sensitive animals.

The 5HT2A receptor is coupled to a protein Gq, activating a phospholipase C (PLC) that produces a mobilization of intracellular calcium and activation of protein kinase C (PKC) and neuronal excitability [127]. Activation of PVN 5HT2A receptors by specific agonists produces an increase in the secretion of hormones related to the stress system, like ACTH, corticosterone, oxytocin, renin, and prolactin, as well as an activation of CRH- and oxytocin-expressing neurons [128]. Increased 5HT2A receptor expression has been reported in individuals with reduced serotonergic tone, as is the case for obsessive–compulsive disorder patients [129] and suicide victims [130] and in anorexia nervosa [131]. In addition, the 5HT2A receptor expression is downregulated after serotonin or antidepressant treatment [132–134]. Taken together,

these reports suggest that a decrease in serotonergic input is related to 5HT2A receptor upregulation. Stress-sensitive animals exhibited lower serotonergic activity as well as fewer serotonin cells and lower expression of genes related to serotonin function in the dorsal raphe nucleus. Therefore, the higher expression of the 5HT2A receptor mRNA observed in stress-sensitive monkeys in the present study may be a compensatory mechanism for the lower dorsal raphe serotonergic tone to the PVN and it may be contributing, in part, to the increased sensitivity to stress observed in these animals.

The dense localization of the 5HT2C receptor in the infundibulum is consistent with our previous observations. Moreover, the 5HT2C receptor was decreased by ovarian steroid hormone replacement in the infundibular nucleus [112]. In this study, we found that the 5HT2C receptor was increased in stress-sensitive animals, which also have the lowest amount of serotonergic tone and the lowest concentrations of estrogen and progesterone during normal menstrual cycles. Either of these deficits could have led to upregulation of the 5HT2C receptor in the infundibulum, a region with a large concentration of neurons that express estrogen and progestin receptors [120].

Like the 5HT2A receptor, the 5HT2C receptor is coupled to a protein Gq, activating the PLC and PKC systems and increasing neuronal excitability [135]. It was recently reported that depletion of serotonin increases expression of 5HT2C mRNA isoforms encoding receptors with higher sensitivity to serotonin [136]. In addition, repeated administration of 5HT2C agonists downregulates 5HT2C receptors [137]. These results suggest that serotonin levels affect the expression and activity of the 5HT2C receptor. Therefore, we speculate that the lower levels of serotonin in stress-sensitive monkeys, together with the lower levels of estrogen and progesterone, contribute to the upregulation of 5HT2C receptor observed in the present study.

GAD67 mRNA was robustly expressed in the infundibulum of cynomolgus macaques, which is consistent with our previous report of GAD67 expression in pigtail macaques. Moreover, we previously found that GAD67 mRNA expression was decreased by ovarian steroid hormone replacement and that 5HT2C receptor mRNA colocalizes with GAD67 mRNA in infundibular neurons [117]. In the present study, we found that stress-sensitive monkeys expressed higher levels of GAD67 mRNA in the infundibular region, compared with highly stress-resilient animals. Moreover, there was a positive correlation between GAD67 and 5HT2C receptor mRNA.

GABA has been proposed as a mediator in the steroid feedback that modulates GnRH secretion [138], and it may play a role in some forms of hypothalamic infertility, like in the polycystic ovarian syndrome, as well as in infertility related to negative energy balance [139, 140]. Increased GAD67 and GAD65 expression is observed in long-term feed-restricted male rats and is involved in the reduction of LH secretion observed in these animals [141]. There is evidence that serotonin inhibits LH secretion in rats through the stimulation of GABA neurons [116]. A body of literature suggests that GABA acts through $GABA_A$ receptors within the vicinity of the GnRH neuron soma to inhibit LH secretion [142–145]. Furthermore, GnRH neurons express functional $GABA_A$ receptors that may inhibit GnRH neuronal excitability [114, 146, 147].

It is attractive to speculate that the lower levels of ovarian steroids in stress-sensitive monkeys and the higher levels of 5HT2C may be responsible for the increased levels of GAD67 observed in stress-sensitive animals. In turn, this could lead to an increase in GABA synthesis and secretion. GABA may be suppressing GnRH and LH secretion, which would affect estrogen and progesterone secretion by the ovary, as indicated by the negative correlation between GAD67 expression and luteal phase progesterone levels obtained in the present study.

In summary, we found that in non-stressed conditions, monkeys that were previously characterized as stress sensitive exhibited higher expression of 5HT2A receptor mRNA in the PVN, an area strongly implicated in governance of neuroendocrine stress responses. Stress-sensitive monkeys also exhibited higher 5HT2C receptor and GAD67 mRNAs in the infundibulum, an area crucial for ovulation. GABA neurons in the infundibulum express steroid hormone receptors and may directly respond to the lower levels of estrogen and progesterone in stress-sensitive monkeys. Alternatively, the lower serotonin tone in stress-sensitive animals may lead to upregulation of 5HT2A and 2C receptors, upregulation of GAD67, a decrease in pituitary LH, and ultimately lower serum estrogen and progesterone levels. However, 5HT1A receptor mRNA in the VMN was not different between the stress-characterized groups. The VMN has been implicated in the regulation of sexual behavior and food intake, but not neuroendocrine function. This further suggests that the serotonin and GABAergic systems may be selectively altered in the stress-sensitive and reproductive-related circuits of stress-sensitive monkeys and may be participating in altering the sensitivity of the reproductive system to stress in these individuals, although non-stress-related circuits may be unaffected. Other regulatory neural systems that are activated in conditions of stress and that impinge upon GnRH neurons include corticotrophin-releasing hormone (CRH) and β-endorphin, derived from pro-opiomelanocortin.

Hypothalamic Systems: CRH and POMC (β-Endorphin)

Introduction

In many stressful situations the hypothalamic–pituitary–adrenal axis becomes activated and CRH, ACTH, and cortisol increase. CRH was originally isolated from the hypothalamus [148] and its neuroendocrine role in the HPA response to stress is well characterized [149, 150]. CRH also plays a role in the integration of autonomic and behavioral responses to stress. Within the limbic system, there is evidence that the CRH system modulates behavioral traits such as locomotor activity, sleep, addictive behavior, and in particular, anxiety-related behaviors [151, 152]. Moreover, CRH neurons and fibers are found in numerous limbic structures outside of the hypothalamus [153–155]. Therefore, we questioned whether CRH expression differed between individuals who show differential sensitivity to stress-induced reproductive dysfunction.

Another neuropeptide involved in the regulation of ovulation is β-endorphin, which is derived from pro-opiomelanocortin [156, 157]. In rodents, the synthesis of β-endorphin is linked to the transcription of POMC mRNA [158]. β-Endorphin-containing neurons inhibit GnRH neuronal activity under certain circumstances, in particular during the progestin-dominated luteal phase of the menstrual cycle [159]. Stress-sensitive animals cease ovulation immediately upon exposure to stress, which could potentially involve rapid activation of POMC neurons and release of β-endorphin. Therefore, we speculated that β-endorphin expression might differ between individuals with sensitivity versus resilience to stress-induced reproductive dysfunction.

In the following study, we examined the expression of CRH in the hypothalamic paraventricular nucleus, the centrum medianum–subfascicularis complex of the thalamus (CM-Sf), and in the central nucleus of the amygdala; and we examined the expression of POMC, the precursor mRNA for β-endorphin, in the medial basal hypothalamus of cynomolgus monkeys that had been previously shown to exhibit different sensitivities to stress-induced reproductive dysfunction.

Methods

CRH mRNA expression was examined with in situ hybridization (ISH) at five levels of the hypothalamic paraventricular nucleus and at five levels of the centrum medianum–subfascicularis complex of the thalamus (CM-Sf). CRH protein expression was examined with immunocytochemistry at five levels of the hypothalamic PVN and at four levels of the central nucleus of the amygdala. POMC mRNA expression was examined with ISH at six levels of the hypothalamic infundibular nucleus. Protocols for immunocytochemistry, in situ hybridization, image analysis with NIH Image, and statistical analysis were previously published [160].

Results

Representative autoradiographs of CRH mRNA signal in the PVN and CM-Sf complex from HSR, MSR, and SS individuals are shown in Fig. 11. The CRH signal is robust in both areas of the SS animals and declines to nearly undetectable in the HSR animals. As shown in Fig. 12, top and middle panels, CRH-positive pixel area and OD were significantly elevated in the caudal two levels of the PVN in the MSR and SS animals compared to HSR animals. There was no difference between groups in the rostral three levels, which masked the difference in the caudal levels in the overall average. Nonetheless, the average of levels 4 and 5 demonstrated a significant difference between the groups ($p<0.05$). Upon examination of the PVN in hematoxylin-stained sections, it appeared that the PVN was larger and extended further in a caudal direction in the MSR and SS groups (not shown). Figure 12, bottom panel, shows that there was a significant positive correlation between the expression of CRH mRNA in the PVN and 5HT2A mRNA in the PVN ($r^2=0.4233$; $p=0.02$). CRH mRNA expression in the CM-Sf of the thalamus was also higher in stress-sensitive animals [160].

The differences in CRH mRNA expression in the PVN were manifested at the protein level as well. Figure 13 contains representative montages of the CRH immunostaining at levels 2 and 4 in the PVN of HSR, MSR, and SS individuals. At matching levels of the PVN, the CRH immunostaining is darker and more widespread in the MSR and SS individuals than in the HSR individuals. To quantify the immunostaining, Slidebook 4.2 was employed to segment positive immunostained pixels. As shown in Fig. 14, left panel, there was a significant increase in the overall average CRH-positive area/section from all five levels in MSR and SS groups compared to the HSR group ($p<0.05$). The percent of the regional volume occupied by CRH immunostaining is shown in Fig. 14, right panel. There was a significant increase in CRH volume in the MSR and SS groups compared to the HSR group ($p<0.05$).

Immunostaining of the amygdala for CRH revealed a dense fiber plexis in the central nucleus. As shown in Fig. 14, bottom panel, there was a significant difference in CRH-positive pixel area between the groups, and the pixel area of CRH fiber staining was significantly higher in the SS group than in the MSR or HSR groups ($p<0.05$).

There was no difference in POMC mRNA expression in the infundibular nucleus of the mediobasal hypothalamus as determined with in situ hybridization.

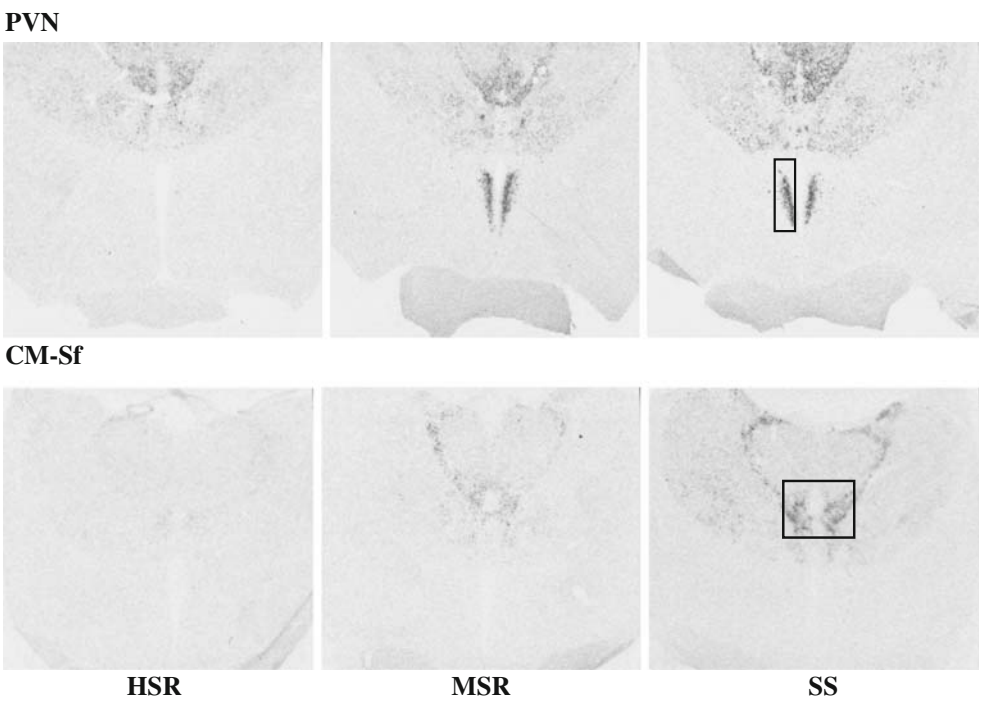

Fig. 11 Autoradiographs of CRH mRNA signal at level 2 of the PVN and CM-Sf of a representative animal from each experimental group (HSR, MSR, and SS). CRH mRNA expression is barely detectable in the HSR animal, but it increases markedly in the MSR and SS animals. Reprinted from Centeno et al. [160]

Discussion

The CRH system has been strongly implicated in stress-related disorders [161]. Moreover, CSF CRH is elevated in individuals with major depressive disorder [151, 162] and in individuals who suffer from adult affective disorders due to childhood trauma and concurrent stress [163, 164].

These data suggest that ongoing CRH production is higher in SS animals in several areas within the central nervous system. Whether this would translate to a greater release of CRH under stress is unknown. Female rats have higher CRH mRNA expression than males, but they do not release more CRH under stress [165]. Moreover, the areas in which CRH was higher are not directly involved in the regulation of the HPA axis. The rostral regions of the PVN contain the neuroendocrine CRH neurons, i.e., the neurons that project to the median eminence and control the secretion of ACTH [166]. The neurons that project to other areas of the brain, particularly to the midbrain and periaquaductal gray regions are present in the caudal two-thirds of the PVN [167, 168]. The CRH produced in the amygdala and CM-Sf of the thalamus probably does not directly regulate ACTH either. Rather, these neurons play a role in communication within the greater limbic system. Outside of the neuroendocrine CRH system, CRH administration produces hypertension, tachycardia, and an elevated oxygen consumption; induces a reduction in food intake; increases grooming behavior, locomotor activity, and vocalization; and induces an aroused state, but decreases sexual receptivity [169]. CRH can act at sites distant from release via convection through CSF [170], and CSF CRH levels are elevated in monkeys with a fearful temperament [171].

Previous studies have shown that CRH and activation of the CRH system inhibit LH secretion and in turn, ovulation, via CRH-R2 receptors [8, 172–174]. Also, CRH fibers have been shown to be juxtapositioned on GnRH neurons in humans [175]. However, stress suppressed reproductive function in CRH knockout mice similar to wild-type mice [176]. In addition, CRH from the PVN did not suppress pulsatile LH secretion in lactating rats [177] and neuroanatomical studies do not find direct connections between CRH and GnRH neurons in rats [178]. We have preliminary data suggesting that there is little difference in the diurnal release of cortisol or the cortisol response to acute stress between SS, MSR, or HSR monkeys [179]. This information leads to the speculation that the lack of difference in CRH expression in the rostral PVN may underlie the lack of difference in normal diurnal HPA axis activity or the HPA axis activation in response to acute stress in monkeys that differ in responsiveness to stress-induced reproductive dysfunction. Instead, the elevation in CRH expression in the caudal PVN, thalamus, and amygdala of SS animals may be communicating with other brain areas.

Our observation of more CRH neurons in the caudal region of the PVN in SS animals is consistent with the report that depressed patients exhibit 4 times more CRH neurons than controls [180]. Although careful volumetric studies are needed, it appears that the PVN extends further in the caudal direction in the SS animals. This observation

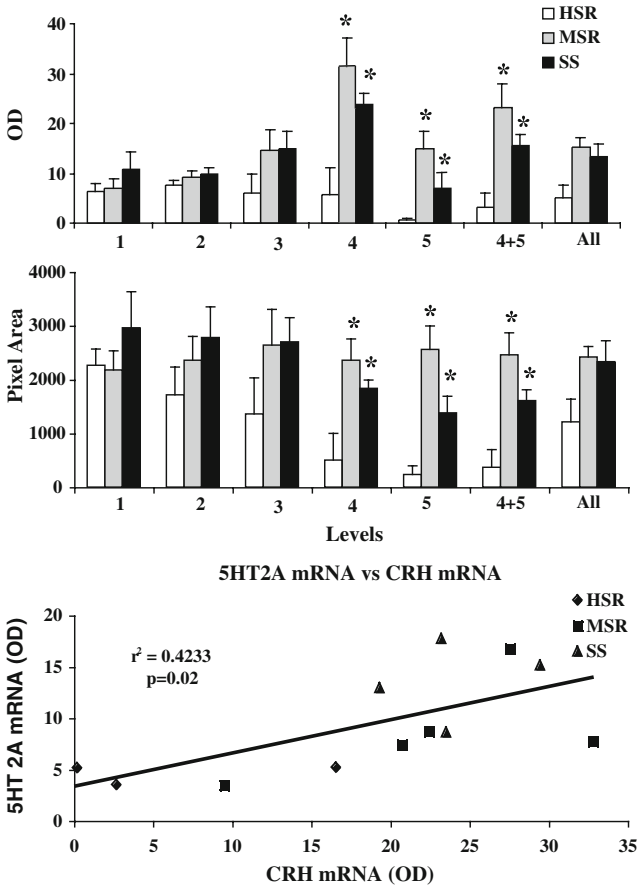

Fig. 12 *Top* and *middle*. Optical density and positive pixel area of the CRH mRNA signal in the PVN at each of the five rostral to caudal levels, in levels 4 and 5 combined, and in all levels combined. There is a significant difference between the groups at caudal levels 4 and 5 and in the average of levels 4 and 5 ($p<0.05$, ANOVA in both analyses). CRH mRNA expression is significantly higher in the MSR and SS groups compared to the HSR group ($p<0.05$, SNK). *Bottom*. The average CRH mRNA expression in the PVN was significantly correlated with 5HT2A mRNA expression in the PVN. Reprinted from Centeno et al. [160]

is supported by an earlier study reporting a larger PVN in depressed patients [164]. The caudal PVN CRH neurons may play a role in the regulation of the serotonin system, which is compromised in SS animals.

The amygdala is a pivotal site of extrahypothalamic production of CRH, and CRH fibers, which project to the bed nucleus of the stria terminalis, are prominent in the central nucleus of the amygdala [181]. The central nucleus plays a crucial role in mediating fear and anxiety in primates [182] and an oral CRH-R1 antagonist decreases stereotypical mouth movements in a behavioral test of anxiety in macaques [183]. Also, infusion of a CRH-R1 antisense oligonucleotide into the central nucleus has been shown to reduce anxiety behaviors in rats [152]. Moreover, the amygdala sends a CRH projection to the dorsal raphe [184], which regulates serotonin neurons [185].

CRH and the urocortins influence several aspects of serotonergic neurotransmission, including the release and synthesis of serotonin as well as the firing rate of serotonin neurons [186, 187]. Knockout of CRH-R1 results in enhanced synthesis of serotonin, suggesting that CRH decreases serotonin through the R1 receptor [188]. It was recently reported that blocking CRH action in the median raphe nucleus decreased c-fos expression in the central nucleus of the amygdala, further emphasizing the reciprocal connectivity of these regions [189]. Thus, our observation that CRH is higher in SS animals than in MSR or HSR animals suggests that there would be an increase in CRH release in the raphe nucleus, which could decrease serotonin function, leading to dysfunction of numerous autonomic systems, including reproduction.

CRH mRNA was elevated in the CM-Sf of the thalamus of SS animals. This area corresponds to the center median-parafascicular complex in humans. A body of literature suggests that CRH neurons in the CM-Sf complex may participate in processing somatosensory and visceral information related to stress [190, 192–194].

Another important neuropeptide that inhibits GnRH neurons is β-endorphin [195, 196]. Moreover, the suppressive effect of CRH on LH secretion is abolished by the opiate antagonist, naloxone, indicating that CRH increases β-endorphin, which in turn, inhibits LH secretion [40]. Therefore, we hypothesized that SS animals may have elevated expression of POMC mRNA, which codes for the precursor protein of β-endorphin. However, the data render this hypothesis null. There was no significant difference in POMC mRNA expression between the SS, MSR, and HSR groups. Nonetheless, we cannot rule out a difference in post-translational processing of the POMC protein, which could lead to differences in the release of β-endorphin peptide. Also, this observation does not rule out differences in the release of β-endorphin under stress, and we are currently examining this possibility.

In summary, under non-stressed conditions, animals that have previously exhibited increased sensitivity to the suppression of reproductive function during stress have significant differences in their brains compared to animals that maintain reproductive function during stress. They have fewer serotonin neurons and reduced expression of serotonin-related genes; they have higher expression of 5HT2A and 5HT2C receptor mRNA in hypothalamic terminal fields; they have higher expression of GAD67 in GABA neurons adjacent to GnRH neurons; and they have higher levels of CRH mRNA and protein in the caudal PVN, higher levels of CRH mRNA in the CM-Sf, and a greater density of CRH fibers in the central nucleus of the amygdala. However, they show no difference in 5HT1A mRNA expression in the hypothalamic ventromedial nucleus, which is not a neuroendocrine area, and they

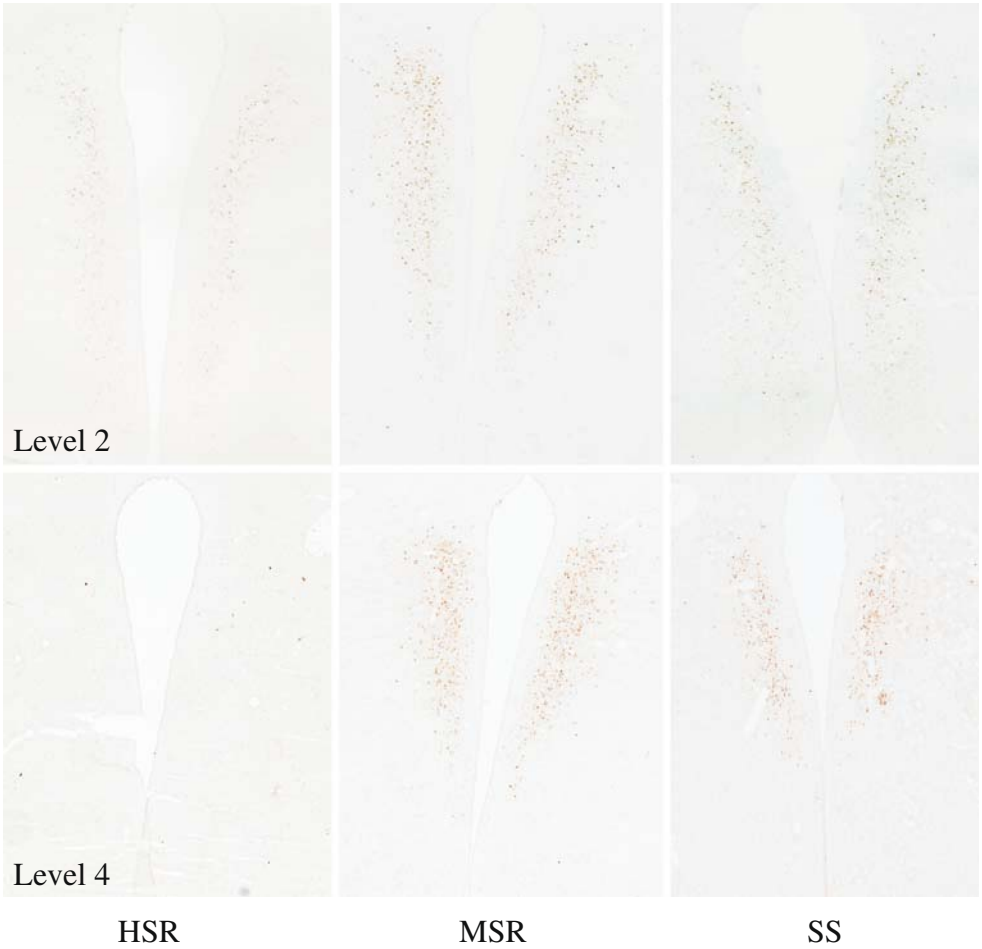

Fig. 13 Photomicrographs of CRH immunostaining at level 2 and level 4 of a representative animal from each group. The immunostaining is darker and somewhat more extensive in the MSR and SS animals at level 2. At level 4, the PVN has disappeared in this HSR animal, but CRH-positive neurons are still prevalent in the MSR and SS animals. By level 5, the PVN was absent in all HSR animals. Reprinted from Centeno et al. [160]

show no difference in POMC expression in the arcuate/infundibular region where GnRH neurons reside in macaques. Our next goal was to examine the GnRH system, the final link in the control of reproductive function.

Hypothalamic GnRH Neuron Function

Introduction

GnRH is the neuropeptide that controls the secretion of pituitary LH and FSH, which in turn regulate the production of ovarian estrogen and progesterone and promote ovulation. The regulation of GnRH is complex, involving both negative and positive feedback through estrogen. Negative feedback involves estrogenic suppression of GnRH, thus removal of the ovaries results in elevated LH. Positive feedback involves the ability of high levels of estrogen to stimulate GnRH, thus producing the preovulatory surge of LH. However, estrogen may act on GnRH neurons via ERβ and act on upstream neural circuits, which impinge on GnRH neurons to achieve the different regulatory components [197]. Since ovulation is the endpoint by which we determined stress sensitivity, and since GnRH is the pivotal neuropeptide governing the HPG axis, we questioned whether the GnRH system was also showing suboptimal function in SS monkeys. To do this, we examined the expression of GnRH mRNA by in situ hybridization and the expression of GnRH peptide by immunohistochemistry in the mediobasal hypothalamus of SS and HSR monkeys.

Methods

Only three animals per group had complete representation of the median eminence and were considered for this analysis. GnRH riboprobe preparation, in situ hybridization, GnRH immunocytochemistry, and image analysis were previously described [198]. Student's *t* test was used for statistical analysis.

Results

Figure 15a and b shows autoradiograms of GnRH ISH signal in the mediobasal hypothalamus of one representative HSR and one SS monkey, respectively. Because GnRH

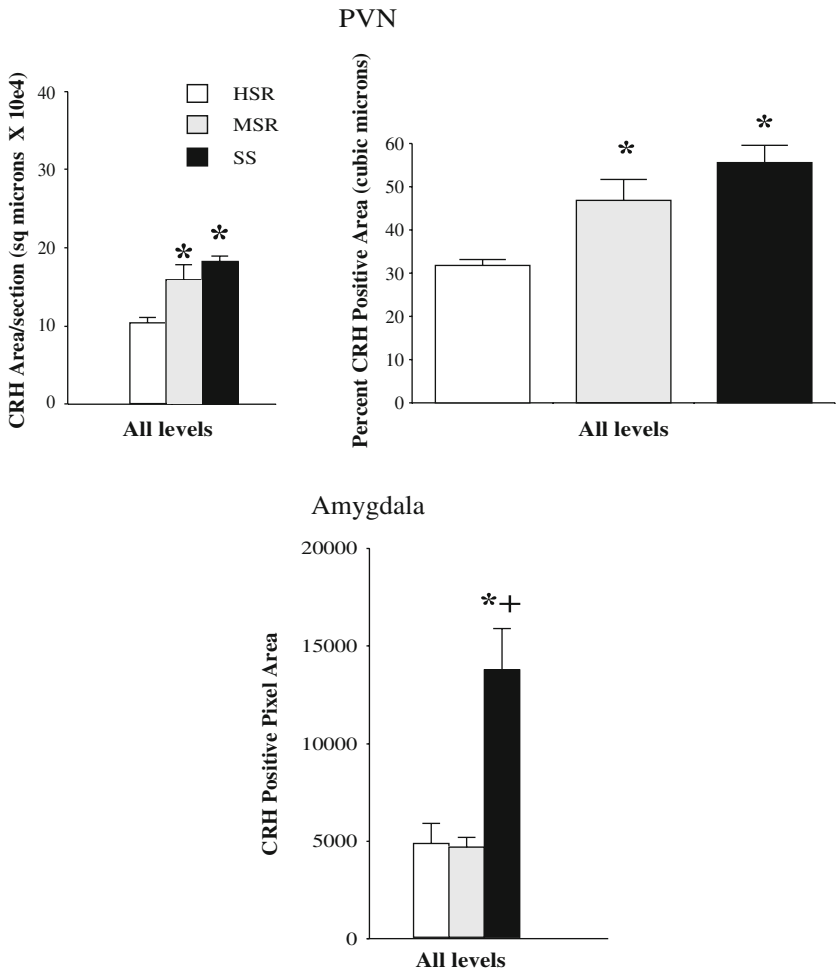

Fig. 14 Histograms illustrating the quantity of CRH immunostaining in five levels of the PVN and in four levels of the central nucleus of the amygdala of the HSR, MSR, and SS groups. *Top left panel* shows the overall average CRH positive area (μm^2)/section in each group. There was a significant difference between the groups with MSR and SS groups exhibiting significantly higher expression of CRH protein than the HSR group. *Top right panel* shows the volume of CRH immunostaining expressed as a percent of the volume of the region (μm^3). There was a significant difference between the groups with MSR and SS groups exhibiting significantly higher expression of CRH protein than the HSR group. *Bottom panel* illustrates the density of CRH fibers (positive pixel area covered by fibers) in the central nucleus of the amygdala from HSR, MSR, and SS groups. There was a significant difference between the groups with the SS group exhibiting significantly higher CRH fiber density than the MSR and HSR groups. *Different from HSR; +different from MSR; ANOVA followed by Student–Newman–Keuls post hoc pairwise comparison. Reprinted from Centeno et al. [160]

cells are diffusely located in the mediobasal hypothalamus, the type of discrete signal observed in the autoradiogram cannot be subjected to densitometry film analysis. Therefore, the sections were exposed with emulsion and individual cells, as well as the number of grains per cell expressed as positive pixels, were counted in the dark field images as shown in Fig. 15c and d. As illustrated in Fig. 16, top and middle panels, SS macaques exhibited more GnRH neurons compared with HSR animals ($p<0.05$). In addition, SS animals exhibited significantly more neurons with 501–1,000 positive pixels per cell and with greater than 2,001 positive pixels per cell (*$p<0.05$).

GnRH-immunoreactive cells were counted in a montage of the arcuate nucleus and the median eminence using the Marianas Stereology workstation and the Slidebook 4.1 software. The number of GnRH-positive neurons per cubic millimeter was calculated and the results are shown in Fig. 16, bottom panel. SS animals exhibited significantly more GnRH-positive cell bodies than HSR animals. Due to the 250-μm distance between the sections, it is unlikely that a GnRH cell body would be present in two sections.

The median eminence was well represented in three SS animals and in three HSR animals, with each animal exhibiting intact median eminence in two to four sections. Individual GnRH-beaded fibers were prominent in the median eminence and were segmented into positive pixels by NIH Image software. As shown in Fig. 17, HSR animals

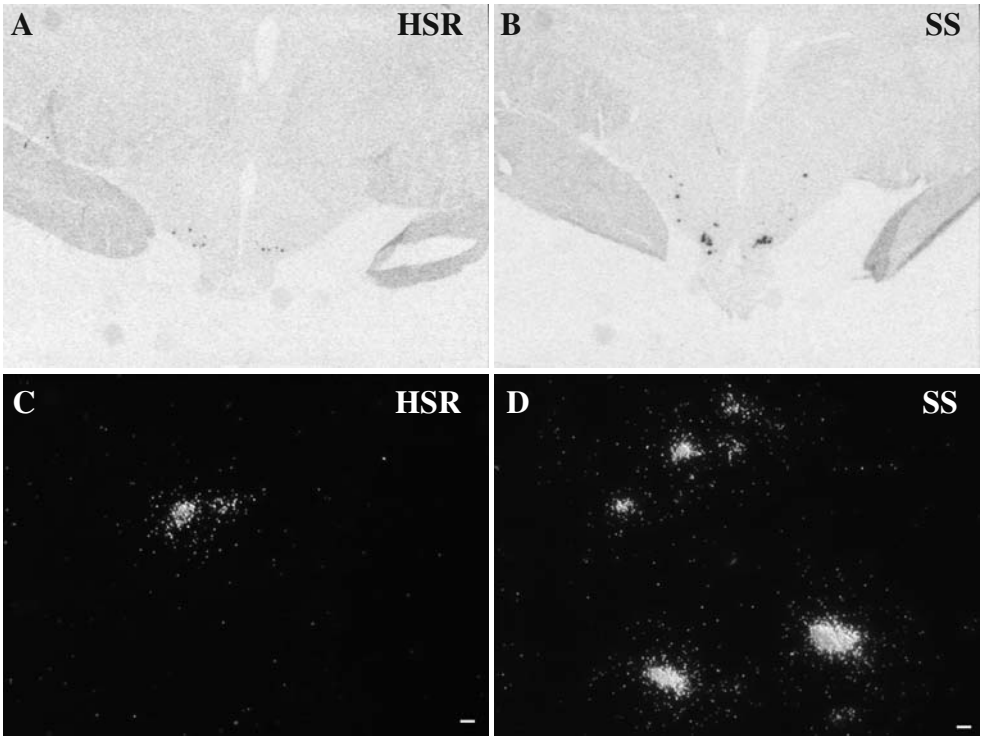

Fig. 15 GnRH mRNA expression in the MBH of high stress-resilient and stress-sensitive monkeys. **a** Autoradiogram of GnRH in situ hybridization signal in the arcuate nucleus and median eminence of one high stress-resilient (*HSR*) monkey. **b** Autoradiogram of GnRH in situ hybridization signal in the arcuate nucleus and median eminence of one stress-sensitive (*SS*) monkey. **c** Dark field image of GnRH ISH signal after exposure with emulsion in the ARC of one high stress-resilient (*HSR*) monkey (scale bar= 10 μm). **d** Dark field image of GnRH in situ hybridization signal after exposure with emulsion in the arcuate nucleus of one stress-sensitive (*SS*) monkey (scale bar=10 μm). Reprinted from Centeno et al. [198]

had a significantly greater number of pixels representing GnRH fiber immunostaining than did SS animals ($p<0.05$).

Of note, the average cycle length for the 8 months preceding euthanasia was obtained for each animal. Each animal had exhibited six or more menses in the 8-month period preceding euthanasia. The three animals in the HSR group exhibited cycle lengths of 31.8±0.6, 31.5±4.0, and 28.9±0.2, respectively. The three animals in the SS group exhibited cycle lengths of 38.2±9.3, 34.0±4.02, and 34.4± 1.3, respectively (not different between groups).

Discussion

In our model of hypothalamic amenorrhea, the endpoint of our stress assessment is ovulation, which is dependent upon proper function of the HPG axis. The pivotal peptide controlling this axis is GnRH. Therefore, we questioned whether the GnRH system was altered in SS versus HSR individuals, either with regard to synthesis of GnRH or secretion of GnRH. We hypothesized that both synthesis and secretion may differ between SS and HSR groups or that synthesis may be unimpaired in SS animals but that GnRH secretion could be impaired as a result of differences in neural input to GnRH neurons.

In the present study, we found that the mediobasal hypothalamus of SS monkeys during the early follicular phase of a non-stressed menstrual cycle (before day 5), exhibited an increased number and density of detectable GnRH cell bodies compared to HSR individuals. In addition, the numbers of GnRH neurons with very high GnRH expression were significantly increased in SS animals compared to HSR animals. Lack of detection of GnRH mRNA or protein in soma expressing low levels of GnRH may be the cause of fewer numbers of GnRH neurons in the HSR animals. Regardless of whether there are more GnRH neurons or simply more highly expressing GnRH neurons in SS animals, clearly, SS animals have higher overall GnRH mRNA and protein in GnRH cell soma. However, SS monkeys showed a significantly lower density of GnRH-positive fibers in the median eminence suggesting that (a) either the peptide accumulated in the cell bodies and was not transported down the axons or (b) that SS animals were producing and releasing more GnRH, thereby depleting the GnRH fibers. As discussed below, we have evidence to support the first explanation, rather than the second. Moreover, we speculate that with stress, these differences become even more exaggerated.

Earlier studies have reported similar changes in GnRH neurons after stress exposure in rats and sheep. Intermittent electric shocks suppressed GnRH secretion and ovulation in ewes [199]. The hypothalamus of those animals showed a reduction of GnRH-immunoreactive fibers in the median eminence, but an increased density of GnRH-immunoreactive cell bodies in the preoptic area [199]. The same result was obtained after nutritional stress [200] or CRF treatment [174]. Moreover, increased activity of the amygdala reduced the density of hypothalamic GnRH-immunoreactive fibers in rats [201, 202]. All of

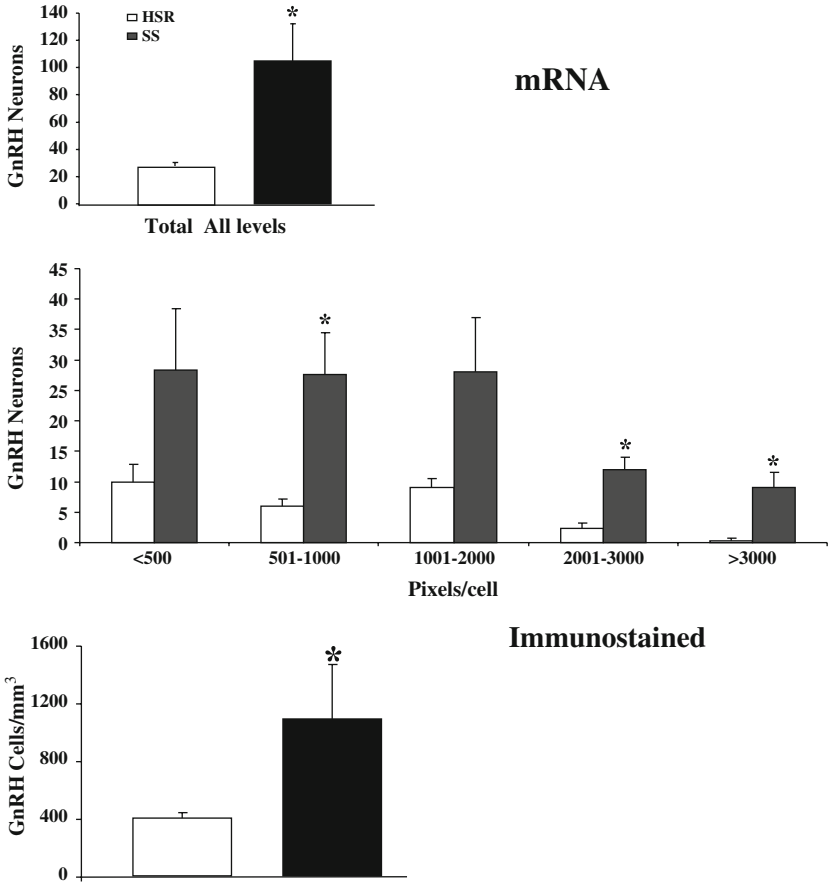

Fig. 16 Analysis of the total number of cells expressing GnRH mRNA in the arcuate nucleus and median eminence of HSR and SS monkeys (n=3/group). *Top*. The number of neurons expressing GnRH mRNA grains at 3× background was counted in six sections through the arcuate nucleus and median eminence. The histogram illustrates the mean (±SEM) of the total number of neurons that express GnRH mRNA. *Middle*. The grain over each GnRH neurons was segmented into positive pixels. The histogram illustrates the mean (±SEM) of the total number of GnRH neurons exhibiting different numbers of positive pixels over grains per cell. *Bottom*. Histogram of the mean (±SEM) density of GnRH-immunopositive cell bodies in the arcuate nucleus and median eminence of HSR and SS monkeys (n=3/group) expressed as the number of cells per cubic millimeter. Six sections at 250 μm intervals through the mediobasal hypothalamus were counted and the total number of neurones was obtained for each animal/mm^3 (density). The group means were then computed and statistically compared. *p<0.05, Student's t test. Reprinted from Centeno et al. [198]

these reports suggested that reduced GnRH transport might be responsible for the higher concentration of the peptide in the cell bodies, but lower density in median eminence fibers, as we observed in SS monkeys.

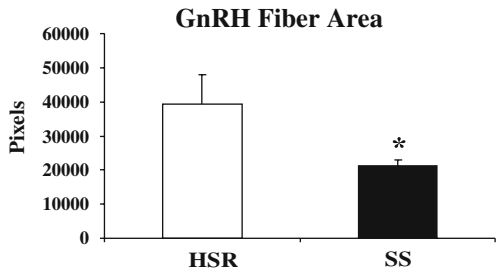

Fig. 17 Histogram illustrating the average of the positive pixels in a sample area of GnRH fibers in the median eminence of HSR and SS monkeys. (n=3/group; *p<0.05, Student's t test). Reprinted from Centeno et al. [198]

There are other important physiological states in which there is discordance between hypothalamic GnRH and LH secretion. For example, LH is high in neonatal monkeys and then becomes suppressed in juvenile monkeys until the onset of puberty. However, there is no difference in hypothalamic GnRH mRNA or protein when neonatal and juvenile male or female macaques have been compared, although pituitary and serum LH are markedly reduced in juveniles [203–205].

The observed association between lower peak E production and higher GnRH mRNA in the SS monkeys is also consistent with other reports. Estradiol treatment of gonadectomized macaques reduced GnRH mRNA expression [206], suggesting that the lower secretion of E in the SS animals may contribute to the higher expression of GnRH. Krajewski et al. reported three subtypes of GnRH neurons in cynomolgus monkeys, and estradiol treatment of ovari-

ectomized cynomolgus monkeys reduced GnRH mRNA expression in type I GnRH neurons [207]. Higher levels of GnRH gene expression in type I GnRH neurons were also reported by this same group in postmenopausal women [208]. In the present study, we found significantly more cells with greater than 2,000 GnRH pixels/cell in SS animals, compared with HSR animals. Based on the size, mRNA content, and localization, we speculate that the cells with greater than 2,000 GnRH pixels/cell represent the type I GnRH neurons. Thus, the lower levels of E production in the SS monkeys may contribute to the higher levels of GnRH mRNA expression observed in this study. In turn, dysregulation of transport could result in decreased GnRH release; decreased LH and FSH release, and compromised ovarian estradiol secretion, thereby establishing a minimally effective HPG axis.

Alternatively, the GnRH neurons of the SS individuals may be producing and secreting more GnRH which would also be manifested by higher GnRH mRNA and lower GnRH fiber content. We are currently conducting studies of pulsatile LH secretion in monkeys with different sensitivities to stress, and our preliminary data indicate that during an 8-h period in the follicular phase, the SS animals have fewer than half the number of LH pulses than do the HSR animals [179]. Thus, it is likely that the lower GnRH fiber staining in SS animals is due to reduced transport and release of GnRH.

Although it has been reported that GnRH neurons express ERβ [209], the effects of E on GnRH neurons have been mainly attributed to actions via interneurons. GABA has been proposed as a pivotal mediator in the steroid feedback that modulates GnRH secretion [138, 141]. A body of evidence shows that GABA acts through $GABA_A$ receptors within the vicinity of the GnRH neuron soma to inhibit LH secretion [142, 143], and GnRH neurons express $GABA_A$ receptors that may inhibit GnRH neuronal excitability [146, 147]. Our observation of increased GAD67 is consistent with an inhibitory effect of GABA on GnRH processing.

The CRF system is also a potential candidate mediating the stress-related suppression of the reproductive axis. Activity of the hypothalamic–pituitary–adrenal axis and cortisol are elevated in many stress conditions that are associated with reproductive dysfunction, including functional hypothalamic amenorrhea [23, 210–212]. Antagonism of CRF has been reported to prevent reproductive failure in stressed male rats [172]. However, other studies have found no causality between cortisol or CRF and LH secretion in various stress models [176, 177, 213], and evidence of direct synaptic contact between CRF and GnRH neurons is lacking [178]. In studies underway, we find little relation between basal cortisol or acute stress-induced cortisol and stress sensitivity status (Herod and Cameron, unpublished data).

In summary, we found that monkeys sensitive to stress-induced reproductive dysfunction showed a higher number and density of GnRH cell bodies and a greater number of GnRH neurons with robust GnRH expression, but a lower density of GnRH fibers in the median eminence, compared with stress-resilient individuals. The mechanisms leading to these observations remain to be defined. However, it is possible that the lower preovulatory estrogen surge leads to greater GnRH expression in the soma, but inhibitory neurotransmitters decrease action potentials within GnRH neurons, which may be coupled to peptide transport [214].

Summary

Individual monkeys who exhibit an immediate cessation of ovulation upon exposure to a combined metabolic and psychosocial stress have altered neural expression of pivotal genes in pathways integrating stress and reproductive function. These alterations are present when the individuals are not stressed and probably play a role in the rapid shutdown of the reproductive system when stress is administered. We found that SS animals have compromised serotonin function. They release less serotonin as indicated by prolactin secretion when administered the serotonin-releasing agent, fenfluramine. Upon examination of serotonin-related gene expression in the dorsal raphe nucleus, we found that compared to HSR animals, SS animals express significantly lower amounts of TPH2, SERT, and MAO-A mRNAs with a similar trend in 5HT1A and MAO-B. Moreover, the expression of each of these transcripts is positively correlated with the peak of progesterone during a control, non-stressed menstrual cycle. That is, the higher the peak of progesterone, the higher the expression of each transcript. Gene expression for the serotonin 2A and 2C receptors was increased in SS animals compared to HSR animals, which is consistent with the presumed lower serotonin afferent input to the PVN and infundibular nucleus. However, 5HT1A receptor expression in the VMN, a non-neuroendocrine nucleus, was not different between sensitivity groups. In addition, GAD67 mRNA expression was significantly higher in the infundibulum of SS animals suggesting that inhibitory GABAergic output within this reproductive center is higher in SS animals than HSR animals. Examination of CRH gene and protein expression revealed some remarkable differences between SS and HSR animals. SS animals apparently have a larger PVN that extends further in the caudal direction. In this PVN extension, CRF neurons are prevalent and there is higher expression of CRH mRNA and protein in the PVN due to this extension. The caudal PVN projects to the amygdala and midbrain, but not to the median eminence. Indeed, there were more CRH fibers in the central nucleus

of the amygdala in SS animals compared to HSR animals. Reasoning from this information, it appears that SS animals have chronic excess CRH, which may impact serotonergic and amygdalar functions. No difference was observed in POMC mRNA expression in the infundibulum under non-stress conditions. The GnRH system was the final common pathway leading to cessation of ovulation. Curiously, there were more GnRH neurons detected in the SS animals than in the HSR animals. However, there were fewer GnRH-positive fibers in the median eminence of SS animals. Together the data suggest that GnRH transport is compromised in SS animals, but how this is accomplished requires further investigation. Altogether, this knowledge suggests that there are profound differences in the function of neural systems in stress-sensitive individuals. They appear to be primed for robust physiological and neurobiological responses to stress, which in our model, translates to rapid cessation of reproductive function. Studies are underway to determine gene expression in these systems during stress and to determine if selective serotonin inhibitors or CRH antagonists can prevent the stress-sensitive response of the reproductive system.

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