

## Sensitivity to stress-induced reproductive dysfunction linked to activity of the serotonin system

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**Objective:** To use a nonhuman primate model and determine whether individuals sensitive to stress-induced reproductive dysfunction have lower activity of central serotonergic neurons under nonstressed conditions.

**Design:** The activity of the central serotonergic system was assessed by measuring responsiveness to a fenfluramine challenge (5 mg/kg, IV) in sedated monkeys previously categorized as highly stress resistant (HSR; n = 4; normal menstrual cyclicality through two stress cycles), medium stress resistant (MSR; n = 5; ovulatory in the first stress cycle but anovulatory in the second stress cycle), or low stress resistant (i.e., stress sensitive, SS; n = 4; anovulatory as soon as stress is initiated). To control for differences in pituitary stores of prolactin or ACTH, the animals were subsequently administered a bolus of thyrotropin-releasing hormone (3 µg/kg) plus corticotropin releasing factor (CRF), (3 µg/kg).

**Setting:** Oregon National Primate Research Center, Animal Services Building.

**Patient(s):** Female cynomolgus macaques exhibiting normal menstrual cycles.

**Intervention(s):** Administration of fenfluramine, a serotonin-releasing drug.

**Main Outcomes Measure(s):** Serum concentrations of prolactin (PRL) and cortisol (F).

**Result(s):** Prolactin release in response to fenfluramine was significantly greater in the HSR group compared with the MSR or SS groups. In contrast, cortisol was higher in the SS group compared with the other two groups. Similar responses were not evident after thyrotropin-releasing hormone + CRF stimulation.

**Conclusion(s):** The lower PRL response to fenfluramine in the stress-sensitive animals suggests that stress-sensitive individuals have decreased activity in central serotonergic neurons. However, the F data suggest that the hypothalamic-pituitary-adrenal axis in stress-sensitive individuals is highly responsive to even small increases in serotonin. (Fertil Steril® 2005;83:148–55. ©2005 by American Society for Reproductive Medicine.)

**Key Words:** Serotonin, stress, anxiety, macaques, fenfluramine, prolactin, cortisol

Exposure to stressful stimuli can lead to a variety of secondary diseases such as anxiety, depression, cardiovascular disease, and immune suppression (1). Reproductive dysfunction also occurs with many forms of stress, including psychosocial stress (2), nutritional stress (3–5), participation in vigorous exercise regimens (6), and immune stress (7). Women presenting at infertility clinics with stress-associated reproductive dysfunction have significantly higher than average scores on a number of psychometric tests indicating higher

levels of eating disorders, dysfunctional attitudes about eating, and obsessive attributes (8–11). These studies suggest that there may be a link between stress-related psychiatric disorders and reproductive dysfunction.

Women seeking relief from infertility also tend to have exposure to multiple stressors and there is increasing recognition that in real-life situations individuals are often exposed to multiple stresses at the same time (2, 7). For example, the reproductive dysfunction associated with functional hypothalamic amenorrhea was thought for many years to stem from exposure to psychosocial stresses, but it is now apparent that some of the neuroendocrine abnormalities associated with functional hypothalamic amenorrhea are indicative of metabolic stress (8–14), and that there is a high incidence of eating abnormalities in this patient population (15–17). Moreover, new treatment therapies for functional hypothalamic amenorrhea that target both strategies for cop-

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ing with psychological stress and removal of metabolic stresses look very promising (18).

We have recently developed an experimental nonhuman primate model of hypothalamic amenorrhea in which mild psychosocial stress combined with a mild diet and moderate exercise regimen lead to a suppression of reproductive function (19, 20). Using this monkey model we find that some individuals are sensitive to stress-induced suppression of reproductive hormone secretion, whereas others are stress resilient. Female cynomolgus monkeys are either highly stress resistant (HSR), and maintain normal menstrual cyclicity when exposed to two cycles of combined stress, or medium stress resistant (MSR) and are ovulatory in the first stress cycle, but anovulatory in the second stress cycle, or stress sensitive (SS) and become anovulatory as soon as stress is initiated (19, 20). We have also identified several characteristics of stress-sensitive individuals. They have higher basal heart rates throughout the 24-hour day compared with more stress-resistant animals (21), and they have significantly lower peak  $E_2$  levels in the follicular phase and significantly lower peak P levels in the luteal phase of a normal, nonstressed, menstrual cycle compared with more stress-resistant animals (19).

The serotonin neural system plays a pivotal role in mood and affective regulation, cognition, satiety, and numerous autonomic functions in response to stress (22–25). Decreased activity of the central serotonin system is found in individuals with increased stress sensitivity and anxiety disorders (26–28). In addition, stress impacts serotonin function in a variety of ways depending on the intensity and duration of the stress (29–31). 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 goal of this study was to determine whether there are differences in endogenous function of the central serotonergic system in stress-sensitive vs. stress-resistant animals in the absence of stress. We used fenfluramine administration followed by measurement of prolactin (PRL) and cortisol to assess global serotonin availability (32–37). Fenfluramine causes an immediate release of serotonin through transporter reversal and then blocks serotonin reuptake. Fenfluramine-induced release of pituitary hormones thus reflects the level of endogenous activity in the serotonergic neurons regulating hypothalamic neuroendocrine systems (32, 38).

## MATERIALS AND METHODS

### Animals

All studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center and performed according to federal

guidelines. Thirteen adult female cynomolgus monkeys (*Macaca fascicularis*) were housed in single stainless steel cages in a temperature-controlled room ( $24^\circ \pm 2^\circ\text{C}$ ), with lights on for 12 hours a day (0700–1900). Monkeys were imported in 1993 and approximate ages established by dental examination. At the time of this study, the monkeys were 12–15 years of age. Monkeys were provided with two meals a day at 0930 and 1500. At each meal they received six high protein monkey chow biscuits (no. 5047, jumbo biscuits, Ralston Purina Co., St. Louis, MO; 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 noncaloric 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 primate enrichment program.

### Timing of Studies

Thirteen female cynomolgus macaques were characterized for sensitivity to stress-induced anovulation in 1999 (20) and sensitivity to stress-induced infertility in 2000 (unpublished data). 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/corticotropin releasing factor (CRF) challenge in September 2001.

### Assessment of Stress Sensitivity

Sensitivity of the reproductive axis to stress for each monkey had been tested in a previous study (19), by assessing changes in menstrual cyclicity and reproductive hormone secretion when animals were exposed to 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 unfamiliar animals surrounded them. 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% of each individual's maximum speed (determined for each monkey in the first week of the study) for 1 hour per day, 5 days per week.

The initial study involved a five menstrual cycle design: 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.

Monkeys were classified as either highly stress resistant (HSR;  $n = 4$ ; maintained normal menstrual cycles throughout two menstrual cycles of stress exposure), medium stress resistant (MSR;  $n = 5$ ; were ovulatory in the first stress cycle, but anovulatory in the second stress cycle), or stress sensitive (SS;  $n = 4$ ; become anovulatory as soon as stress was initiated). After termination of the initial stress paradigm, blood samples were obtained through an additional three menstrual cycles under nonstress conditions for determination of peak estrogen (E) and progesterone (P) concentrations. Of note, the original study characterizing stress sensitivity in 1999/2000 was initiated with 15 animals, but 2 animals were euthanized for clinical reasons during the project period.

### Preliminary Test of Anesthesia

Two male rhesus macaques were maintained for 2 hours under either ketamine or propofol anesthesia. Both animals were initially sedated with ketamine (40 mg, IM, Ketaset, Fort Dodge Animal Health, Fort Dodge, IO) in the home cage and transported to the 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. A catheter was inserted into the cephalic vein in the forearm for anesthesia administration. The saphenous vein in the leg was catheterized for blood sampling.

For anesthesia maintenance on ketamine, the animal continued to receive 50 mg IV at 20-minute intervals through the cephalic catheter. For anesthesia maintenance on propofol (Diprivan 1%, AstraZeneca Pharmaceuticals, Wilmington, DE) the animal received five bolus doses of 500  $\mu\text{g}/\text{kg}$  body weight IV, followed by constant infusion at 160  $\mu\text{g}/\text{kg}$  body weight through the cephalic catheter. Heart rate and oxygen flow were monitored continuously. Blood samples were obtained every 10 minutes for PRL and cortisol radioimmunoassay. The blood samples were maintained on ice until the end of the experimental period. They were transported to the laboratory and processed for plasma immediately. Plasma samples were stored at  $-20^{\circ}\text{C}$  until assay.

### Fenfluramine Challenge Protocol

On day 1 of a nonstressed 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. A catheter was inserted into the cephalic vein in the forearm and IV infusion of propofol for anesthesia was

initiated at 160  $\text{mg}/\text{min}/\text{kg}$  body weight and maintained throughout the duration of the experiment with a Harvard pump. The saphenous vein in the leg was catheterized for blood sampling and for administration of the fenfluramine with a short extension tube connected to a three-way stopcock.

When the animals reached deep sedation/light anesthetic condition (usually within 20 minutes after propofol takes effect), blood sampling was initiated. Blood samples (0.5 mL) were obtained every 10 minutes for 2 hours to establish a baseline of hormone secretion. Then, fenfluramine (5  $\text{mg}/\text{kg}$  in saline; Sigma, St. Louis, MO) was injected and blood samples were obtained for an additional 2 hours at 10-minute intervals. After completion of sampling, the catheters were removed by the surgical staff and the animals were awake within 5 minutes of termination of the propofol infusion. Animals were then returned to their home cage and monitored for return of normal activities.

The blood samples were maintained on ice until the end of the experimental period and then processed as described.

### Thyrotropin-releasing Hormone/CRF Challenge Protocol

A control experiment was performed to determine whether responses in the fenfluramine challenge resulted from differential responses of the serotonin system to fenfluramine or differences in pituitary stores of PRL and ACTH in the different groups. In the control study, the animals were administered a bolus of thyrotropin-releasing hormone (Peninsula Lab, Belmont, CA; 3  $\mu\text{g}/\text{kg}$ , IV) plus CRF (Peninsula Lab; 3  $\text{mg}/\text{kg}$ , IV) in the early follicular phase of a subsequent menstrual cycle and blood samples were processed as described.

### Assays

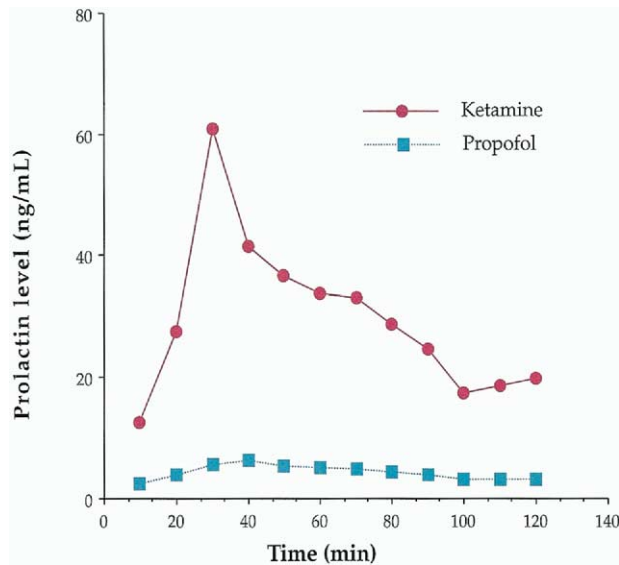
Assays for PRL, cortisol, E, and P were performed using a Roche Diagnostics 2010 Elecsys assay instrument. Each assay had been validated for macaque plasma in comparison with traditional RIA. Interassay coefficients of variation were less than 10% for each assay.

### Statistical Analysis

A two-factor analysis of variance (ANOVA) was conducted on the data with group and time as the dependent variables using the Statistix Analytical Software package (Tallahassee, FL). 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 nonparametric 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  $P < .05$ . Data is presented as mean  $\pm$  SE.

**FIGURE 1**

Plasma prolactin concentrations at 10-minute intervals for 2 hours in animals maintained under ketamine anesthesia ( $n = 1$ ) or propofol anesthesia ( $n = 1$ ). The average prolactin concentration for the 2-hour period was almost six fold higher with ketamine compared with propofol ( $P < .0001$ , Welch's  $t$  test).



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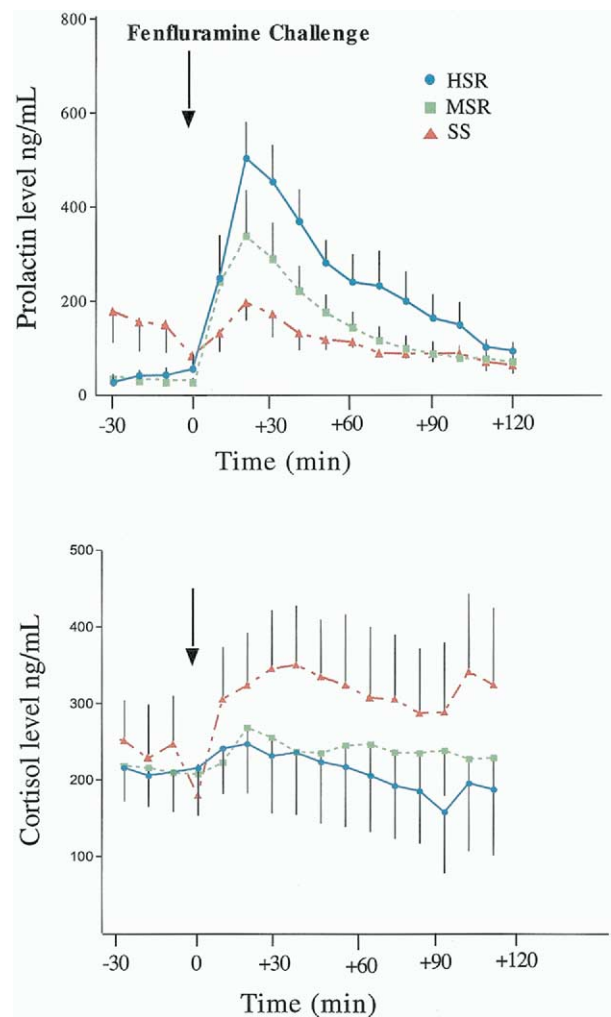
## RESULTS

Under ketamine anesthesia, heart rate varied from 105 to 120 beats/min ( $117.67 \pm 2.72$ ) and under propofol, heart rate varied from 110 to 135 beats/min ( $123.8 \pm 2.11$ ). There was no statistically significant difference between the means ( $P > .08$ ; Student's  $t$  test). Prolactin was significantly elevated under ketamine anesthesia compared with propofol (Fig. 1). The mean of all samples obtained under ketamine equaled  $29.65 \pm 3.8$  and the mean of all samples obtained under propofol equaled  $4.21 \pm 0.35$  ( $P < .0001$ , Welch's  $t$  test for unequal variances). Cortisol varied from 151 to 215 under ketamine and varied from 157 to 294 under propofol (means  $170.62 \pm 5.1$  vs.  $225.55 \pm 14.1$ , respectively,  $P < .003$ ; Welch's  $t$  test). In brief, PRL was nearly six-fold higher under ketamine than propofol, whereas cortisol was only 1.3-fold higher under propofol and heart rate was similar under both anesthetics. Because the PRL response was of greatest interest, propofol was chosen for all further experiments.

As shown in Figure 2, top panel, PRL secretion was significantly different between the experimental groups (two-way ANOVA,  $P < .0001$ ). Prolactin levels before fenfluramine challenge were higher in the stress-sensitive group than in the high or medium stress resistant groups ( $P < .01$ ). However, stress-sensitive animals had a lower response to

**FIGURE 2**

Prolactin (*top*) and cortisol (*bottom*) responses to an injection of fenfluramine (5 mg/kg, IV) 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,  $P < .0001$ , with the HSR group secreting significantly more prolactin compared with the SS group (Tukey's post-hoc test,  $P < .001$ ). In the *bottom* panel, the cortisol response to fenfluramine was significantly different between the experimental groups (two-way ANOVA,  $P < .0001$ ), with the SS group secreting more cortisol compared with the HSR group (Tukey's post-hoc test,  $P < .001$ ).



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fenfluramine compared with high stress-resistant animals (post-hoc test HSR vs. SS,  $P < .001$ ). The PRL response of the medium stress-resistant animals did not differ from the other two groups. Cortisol secretion in response to fenflura-

mine (Fig. 2, bottom panel) was also significantly different between the experimental groups (two-way ANOVA,  $P < .0001$ ; post-hoc test HSR vs. SS,  $P < .001$ ). Stress-sensitive animals had a greater release of cortisol compared with high stress-resilient animals, with the medium stress-resistant animals again showing no significant difference from the other two groups.

In contrast, PRL secretion in response to the thyrotropin-releasing hormone + CRF challenge (Fig. 3, top panel) was not suppressed in the stress-sensitive group, as it was in response to fenfluramine. After thyrotropin-releasing hormone + CRF, prolactin levels were significantly higher in stress-sensitive compared with stress resistant animals ( $P < .01$ ). Basal levels of PRL before challenge were again higher in the stress-sensitive group ( $P < .01$ ). As illustrated in Figure 3, bottom panel, cortisol increased in response to the thyrotropin-releasing hormone + CRF challenge in each group ( $P < .04$ , Friedman's ANOVA), but it was not different between the three groups ( $P > .1$ , two-way ANOVA).

Examination of the ovarian hormone secretion during three control menstrual cycles before this study indicated that the peak E and P concentrations were consistently and significantly higher in the HSR animals than in the SS animals. The follicular E peak averaged  $653 \pm 40.8$  pg/mL in HSR animals vs.  $416.7 \pm 25.6$  pg/mL in SS animals (Student's *t* test,  $P < .008$ ). The luteal P peak averaged  $26.3 \pm 2.4$  ng/mL in HSR animals vs.  $12.5 \pm 1.3$  ng/mL in SS animals (Student's *t* test,  $P < .007$ ).

## DISCUSSION

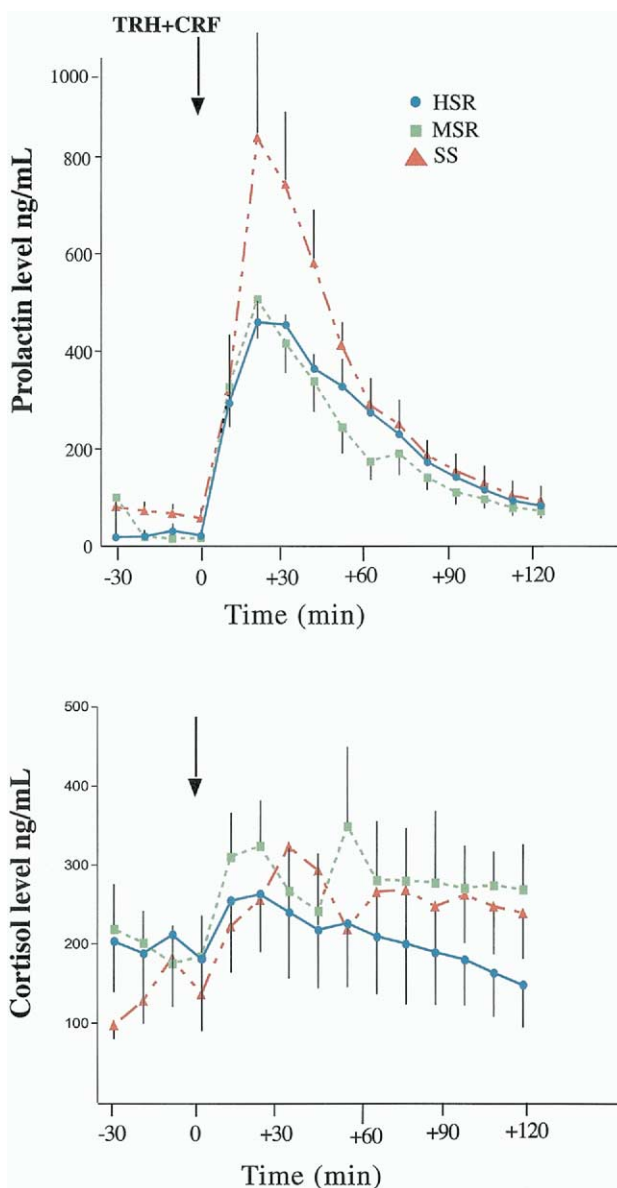
Stress can lead to a variety of secondary disease processes, including reproductive dysfunction in sensitive individuals (1–7, 39). Serotonin appears to be a key neurotransmitter in the regulation of mood, including affective state and anxiety level, and serotonin secretion is decreased in response to stress (24–26). 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 or drug abuse (40, 41). Decreases in ovarian steroid hormones also lead to decreases in various aspects of serotonin neural function (42–44).

Therefore, we questioned whether differences in the activity of the serotonin system could be associated with differences in sensitivity to stress-induced reproductive dysfunction. Macaques have monthly menstrual cycles, like women, as well as highly developed cerebral cortices, and they live in complex social groups. Thus, they are exceptionally good models for understanding the neural underpinnings of stress-induced reproductive dysfunction.

We recently demonstrated that female cynomolgus monkeys show striking individual differences in the response of the reproductive axis to a moderate level of combined psychosocial and metabolic stress (19). About one third of animals showed a rapid and profound suppression of repro-

## FIGURE 3

Prolactin (top) and cortisol (bottom) responses to an injection of thyrotropin-releasing hormone (TRH) ( $3 \mu\text{g}/\text{kg}$ ) + CRF ( $3 \mu\text{g}/\text{kg}$ ) while the animals were maintained under propofol anesthesia. In the top panel, prolactin secretion in response to the TRH+CRF challenge was not suppressed in the SS group. The stress-sensitive animals released the largest amount of prolactin in response to TRH ( $P < .01$ , two-way ANOVA). In the bottom panel, cortisol secretion increased in response to the TRH + CRF challenge in each group ( $P < .04$ , Friedman's ANOVA), but cortisol was not different between the three groups ( $P > .1$ , two-way ANOVA).



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ductive hormone secretion (SS), whereas about one third retained normal menstrual cyclicality throughout 2 months of stress exposure (HSR). The final one third retained menstrual cyclicality during the initial phases of stress exposure but then lost cyclic secretion of reproductive hormones (MSR). The average peak concentrations of E and P across three control menstrual cycles were significantly higher in the HSR than SS animals, with the MSR group showing intermediate levels of these hormones, even before stress was initiated.

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.

Serotonin plays a pivotal role in sensitivity to stress and we questioned whether animals with different sensitivities to stress could have endogenous differences in their serotonin system. Fenfluramine is a serotonin-releasing agent, which causes an increase in PRL or cortisol believed to directly reflect the endogenous availability of serotonin (32, 38, 45). We administered fenfluramine to the previously characterized animals long after they were returned to control, nonstressed conditions and cycling normally. We found that stress-sensitive animals secreted significantly less PRL than stress-resilient animals after an injection of fenfluramine. These data support the idea 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 supports the long-standing notion 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 CRF neurons driving the hypothalamic-adrenal axis are supersensitive to serotonin. Earlier work demonstrated that serotonergic denervation increases the functional neuroendocrine response of the hypothalamic-pituitary-adrenal axis to serotonin agonists (46). Hence, in the stress-sensitive group, even the lower amount of serotonin that was released by fenfluramine may have acted on supersensitive CRF neurons. This line of reasoning is supported by a report that serotonergic stimulation of PRL and corticosterone secretion is mediated by different pathways from the mediobasal hypothalamus in rodents (47, 48). The cellular or molecular mechanism involved in the switch of axis sensitivity between stress-sensitive and stress-resilient animals could be of great interest.

Alternatively, rodent studies suggest that the effect of fenfluramine on corticosterone is not mediated by serotonin release (34). If this is true in primates, then perhaps the nonserotonin-mediated effect of fenfluramine on cortisol is greater in stress-sensitive than in stress-resilient animals. However, the nature of the nonserotonin mechanism remains unresolved.

The control study with thyrotropin-releasing hormone + CRF administration showed that the pituitary stores of PRL are not reduced in stress-sensitive animals. The stress-sensitive animals released more PRL when challenged with thyrotropin-releasing hormone than the other groups, indicating that they have robust pituitary stores of PRL. Thus, we conclude that the release of PRL was dependent on fenfluramine-induced serotonin release, and that more serotonin was released in stress-resilient than stress-sensitive individuals. Unlike stress-resilient animals, stress-sensitive animals exhibited an increase in cortisol in response to fenfluramine that was not due to increased pituitary stores of ACTH. Thus, although stress-sensitive individuals appear to have less endogenous serotonin available for release, their hypothalamic-pituitary-adrenal axis has greater sensitivity to fenfluramine than their PRL axis. In a similar manner, fenfluramine challenges in alcoholic patients produce lower PRL and higher cortisol secretion than in nonalcoholic controls (49, 50).

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 nonstressed animals that had a previously documented difference in reproductive function under stress. We show that there are endogenous differences in serotonin capacity even in the nonstressed 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, therefore 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 (51). A relationship between low socioeconomic status and low serotonergic activity, also measured by the PRL response to fenfluramine, has been observed in a study of men and women (52).

From the earlier studies in which reproductive function was characterized, we know that the stress-sensitive animals have lower peak and lower average E and P levels across three nonstressed menstrual cycles. Ovarian steroid production appears to be a stable endogenous and individual state. Quantitative Trait Locus studies in baboons have found a microsatellite polymorphism that has a significant effect on

E (53). Hence, the apparent decrease in endogenous serotonin in the stress-sensitive animals could be a result of genetic factors leading to lower ovarian steroids across a typical cycle. Moreover, E and P regulate serotonin gene expression in the raphe nucleus of the macaque in a manner that would promote an increase in serotonin capacity (42). It is attractive to speculate that better ovarian function in the HSR animals promotes higher serotonin functional capacity. Further studies on gene expression in the serotonin system of these animals are underway.

In conclusion, cynomolgus macaques exhibit different reproductive responses to moderate stress indicative of high, medium, or low stress resilience. Their stress sensitivity correlates with PRL secretion in response to the serotonin releaser, fenfluramine, suggesting that highly stress resistant animals have higher levels of endogenous serotonin than stress-sensitive animals in the absence of stress. Further study is needed to determine whether this is due to ovarian steroid secretion, social status, or genetic predisposition.

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