Interactions of Corticotropin-Releasing Factor, Urocortin and Citalopram in a Primate Model of Stress-Induced Amenorrhea

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Key Words

Stress • Selective serotonin reuptake inhibitor • Corticotropin-releasing factor • Urocortin I • Dorsal raphe • Serotonin • Macaques

Abstract

Background/Aims: We established a cynomolgus macaque model of stress-induced amenorrhea in which the application of combined metabolic and psychosocial stress suppressed ovulation in stress-sensitive (SS) individuals, but not in highly stress-resilient (HSR) individuals. We previously reported that SS monkeys have deficits in global serotonin release and serotonin-related gene expression in the raphe nucleus, and that administration of the selective serotonin reuptake inhibitor S-citalopram increased estrogen and progesterone production in SS monkeys. In this study, we questioned whether there was a difference in corticotropin-releasing factor (CRF) or urocortin (UCN) stress-related peptide systems in the midbrain raphe region when HSR and SS monkeys treated with placebo or S-citalopram are compared. **Methods:** Monkeys characterized as HSR or SS were administered placebo or S-citalopram for 15 weeks. CRF fibers in the dorsal raphe were detected with an antibody against human CRF. UCN1 fibers were immunostained in an area rostral

to the dorsal raphe. The fibers were quantified by stereology and analyzed by two-way ANOVA. UCN1 cell bodies were counted in the supraoculomotor area near the Edinger-Westphal nucleus. **Results:** S-citalopram significantly decreased the CRF fiber density in SS animals, but not in HSR animals. SS monkeys had a significantly lower UCN1 fiber density compared to HSR monkeys, but S-citalopram treatment did not alter the UCN1 fiber density. SS animals treated with S-citalopram tended to have a higher number of UCN1-positive cell bodies than the other groups. **Conclusion:** S-citalopram decreased CRF fiber density and appears to increase the production of UCN1 in SS individuals, indicating the likelihood that serotonin is involved in regulating CRF and UCN1 in individuals who are sensitive to the effects of serotonin.

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Introduction

International estimates indicate a 9% prevalence of infertility (of 12 months) [1], and approximately 30% of women presenting at infertility clinics in the USA are diagnosed with functional hypothalamic amenorrhea, which has lately been called ‘stress-induced amenorrhea’

[2–4]. We have developed an experimental nonhuman primate model of hypothalamic amenorrhea in which mild psychosocial stress combined with a mild metabolic stress, such as diet or a moderate exercise regimen, leads to a suppression of the reproductive function which is reversed upon stress removal [5–7]. Results from studies of this model have important implications for thousands of infertile women. Using this monkey model, we found that some individuals are sensitive to stress-induced suppression of reproductive function, while others are stress resilient. Female cynomolgus monkeys are either (1) highly stress resilient (HSR) and maintain normal menstrual cycles when exposed to 2 cycles of combined stress, or (2) medium stress resilient (MSR) and are ovulatory in the first menstrual cycle involving stress exposure, but anovulatory in the second stress cycle, or (3) stress sensitive (SS) and become anovulatory as soon as stress is initiated [5, 6, 8].

We have shown that even in nonstressed conditions there are differences in the function of several key neural systems in SS versus SR monkeys. SS animals chronically have a lower release of serotonin (5-HT) in response to fenfluramine [6], and have a downregulation of the central serotonergic system, as indicated by significantly less tryptophan hydroxylase 2 (TPH2) expression [9], less serotonin reuptake transporter (SERT) gene expression, less 5-HT_{1A} receptor gene expression and less expression of the genes that degrade serotonin (MAO-A and MAO-B) in the raphe nucleus [7, 10]. In the hypothalamus, there is an upregulation of 5-HT_{2A} and 5-HT_{2C} receptors [11], and an increase in GAD67 gene expression [11], which are inhibitory to the reproductive axis. We also found that SS animals have less immunoreactive gonadotropin-releasing hormone (GnRH) in the median eminence, lower pituitary luteinizing hormone and follicle-stimulating hormone secretion, and lower ovarian estradiol and progesterone secretion compared to more stress-resilient monkeys, even when they are not experiencing stress [10, 12].

We also found a significant increase in corticotropin-releasing factor (CRF) gene and protein expression in the hypothalamic paraventricular nucleus (PVN) of SS individuals compared to HSR individuals [13]. In many stressful situations, the hypothalamic-pituitary-adrenal axis becomes activated with increases in CRF, adrenocorticotrophic hormone and cortisol, and CRF may inhibit GnRH release [14–16]. In clinical studies, individuals with depression or anxiety or with suicidal tendency exhibited more CRF neurons in the hypothalamus than normal individuals [17, 18]. Moreover, CRF neurons and

fibers are found in numerous limbic structures outside of the hypothalamus, including the dorsal raphe nucleus, and they mediate stress responses [19–21].

Another peptide system involved in the regulation of stress and anxiety is the urocortin (UCN) system, which bears some homology with CRF [22]. Three UCN peptides have been characterized: UCN1, UCN2 and UCN3; both CRF and UCN1 fibers innervate the primate dorsal raphe nucleus [23, 24]. UCN1 is involved in the stress adaptation response [25] and in stress-induced anxiety [26]. Moreover, UCN1 neurons respond to alcohol and may affect alcohol preference and consumption [27]. Evidence from rodents suggests that, once released into the raphe, CRF and UCN1 bind to CRF receptor 1 (CRF-R1) and CRF receptor 2 (CRF-R2) and regulate serotonin neurotransmission [28–31].

Recently, we characterized a new population of monkeys as SS and HSR, and then treated them with the antidepressant escitalopram or placebo for 15 weeks in the absence of stress [32]. We found that there was an increase in peak estradiol and progesterone secretion only in the SS group treated with escitalopram, suggesting that central neural drive to the reproductive axis was improved by escitalopram treatment [32].

Escitalopram is an antidepressant drug used to treat major depressive disorder, generalized anxiety disorder, social anxiety disorder or panic disorder. Escitalopram belongs to a class of drugs known as selective serotonin reuptake inhibitors (SSRI); it is the S-stereoisomer (enantiomer) of the earlier drug, citalopram, and it has the highest affinity for the transporter of any of the SSRI. Thus, how this mechanism of action transmitted to an increase in ovarian steroid secretion became of interest.

The pharmacological actions of S-citalopram indicate that the serotonin system was a primary site of action. Therefore, we examined the expression of 4 pivotal genes that regulate the function of the serotonin neural system. We found that the expression of the master serotonin developmental gene *Fev* (Fifth Ewing variant), as well as that of TPH2, SERT and 5-HT_{1A} mRNA was significantly compromised in SS animals, but that escitalopram treatment for 15 weeks did not alter the expression of these genes [9]. This suggested that the effect of S-citalopram on reproductive function was mediated by a mechanism other than gene expression in serotonin neurons. Because we had observed higher CRF gene and protein expression in the PVN of SS animals, we questioned whether there was a difference in the CRF and UCN1 stress-related peptide projection systems in the midbrain raphe region, and whether S-citalopram altered these parameters.

Materials and Methods

Animals and Treatments

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. Twenty-five female cynomolgus monkeys started the study, but 16 were utilized for neuroanatomical assays in a 2×2 block design with 4 animals per group as explained below. They were housed in single cages, in large rooms with other monkeys. Movement of monkeys within the room, and in and out of the room, was limited so that the monkeys had a stable social environment for 4 months. The monkeys were fed commercially available monkey chow (12 pellets of Purina High Protein monkey chow) once a day at about 11.00 h supplemented with fruit. Water was available *ad libitum*.

Menstrual cycles were monitored by daily swabbing of the vaginal area with a cotton-tipped applicator. The monkeys were trained in the routine of jumping from their home cage into a portable carrying box for transport to a restraint cage, and then extending their leg for blood sample collection by femoral venipuncture. They appeared to readily adapt to the procedure, as evidenced by normal cortisol levels. Blood samples were collected every other day by femoral venipuncture to monitor reproductive hormones throughout the study. To categorize the monkeys as HSR or SS, they were moved to a new housing room, surrounded by unfamiliar monkeys and put on a diet change eating 80% of the food they consumed during the control phase of the study. Their menstrual cyclicity and reproductive hormones were evaluated for 2 months after the move/diet, as previously described [7]. Based upon the menstrual response to this combined psychosocial and metabolic stress, the monkeys were labeled HSR ($n = 8$), MSR ($n = 4$), SS ($n = 7$) and very SS (VSS; $n = 6$). The VSS animals did not exhibit cycles for 4 months of observation in their home cage, and so they were not moved.

At the end of the 4-month period (1 month of control, 1 month of learning to run, 2 months of stress), all of the animals in the paradigm were returned to their home cage and allowed to eat *ad libitum*. Approximately half of each group was treated with vehicle (placebo, Pb) and the other half was treated for 15 weeks with 1.2–4.8 mg/kg of S-citalopram (*b.i.d.*) to elevate blood concentrations of S-citalopram to the therapeutic range for treating depression. Blood samples were collected twice from representative animals to monitor blood citalopram levels by chiral reversed-phase liquid chromatography [33, 34], and doses were adjusted if necessary to keep the levels in the target range of 20–40 ng/ml, as observed in depressed patients. After about 1–2 weeks, blood levels of S-citalopram at 3 and 6 h after dosing averaged 24.8 ± 5.0 and 13.5 ± 3.6 ng/ml, respectively, and the significantly less active metabolite, desmethyl-S-citalopram averaged 12.1 ± 1.7 and 8.7 ± 1.8 ng/ml, respectively ($n = 5$ animals), which required adjustment. After a further 3 weeks, blood levels of S-citalopram at 3 and 6 h after dosing averaged 23.7 ± 6.2 and 22.4 ± 5.7 ng/ml, respectively, and desmethyl-S-citalopram averaged 13.0 ± 3.1 and 13.0 ± 3.1 ng/ml, respectively ($n = 6$ animals). Menstrual cyclicity and reproductive hormones were monitored throughout the S-citalopram treatment as described above.

The S-citalopram treatment was physiologically efficacious, as indicated by an improvement in ovarian steroid secretion [32]. The groups that showed some sensitivity to stress (MSR, SS and

VSS groups) were combined ($n = 17$) and compared to the HSR group ($n = 8$). In SS monkeys, S-citalopram treatment significantly increased peak estradiol levels in the follicular phase of the menstrual cycle from 360 ± 67 to 544 ± 82 pg/ml ($p < 0.05$), and increased peak progesterone levels in the luteal phase of the menstrual cycle from 6.7 ± 1.4 to 11.3 ± 1.8 ng/ml ($p < 0.05$). In contrast, Pb- and S-citalopram-treated HSR monkeys had similar peak estradiol and peak progesterone before and after treatment, which averaged (both groups) 575 ± 100 pg/ml and 11.5 ± 3 ng/ml, respectively. For the subsequent neuroanatomical analysis, only the SS and VSS groups were combined. The MSR animals were not included, as explained below.

Tissue Preparation

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.

The left ventricle of the heart was cannulated and the head perfused with 1 liter of saline (made with diethylpyrocarbonate-treated water, 0.1% diethylpyrocarbonate, to minimize RNase contamination) followed by 7 liters of 4% formaldehyde in 3.8% borate, pH 9.5 [35, 36]. The brain was removed and the pontine midbrain dissected from the rostral to the caudal borders of the pons. The pontine midbrain block displayed the rounded central canal on its anterior surface and the wing-shaped canal on its caudal surface. Blocks of tissue containing the pontine midbrain were postfixed for 3 h in 4% formaldehyde, then washed in 0.02 M potassium phosphate-buffered saline (KPBS) containing first 10% (overnight), then 20% glycerol (48 h), plus 2% DMSO to cryoprotect the tissue. The blocks were frozen in precooled methylbutane (-55°C) and stored at -80°C for up to 6 months.

Post hoc Animal Selection for Tissue Analysis

The 8 HSR animals that were treated with Pb or S-citalopram were all included ($n = 4$ per treatment). The 4 animals that were MSR yielded only 2 each for Pb or S-citalopram treatment, so they were excluded due to insufficient numbers for statistical analysis. The SS and VSS groups were grouped together as stress sensitive. Five SS animals were treated with Pb, but 1 animal had a poor perfusion of the brain, so the remaining 4 animals were included. Eight SS animals were treated with S-citalopram. In order to match the other 3 groups, 4 of the 8 animals, which never cycled or which exhibited immediate cessation of ovulation upon moving, were included in the tissue analysis providing 4 per group in a 2×2 block design. The midbrain blocks containing the Edinger-Westphal nucleus and the dorsal raphe were cut on a sliding microtome at $25\ \mu\text{m}$. Floating sections from the midbrain block were stored in cryoprotectant solution (50% 0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20°C and used later for immunocytochemistry.

Immunocytochemistry

Midbrain sections were removed from -20°C storage and washed in KPBS buffer 4 times for 15 min each, immersed in 0.6% hydrogen peroxide for 30 min, washed in KPBS buffer 4 times for 15 min each and then incubated with the following blocking solutions: Vector normal goat serum (Vector Laboratories, Burlingame, Calif., USA) for 60 min; 3% BSA (Sigma, St. Louis, Mo.,

USA) for 60 min; Vector avidin for 20 min, and Vector biotin for 20 min. Sections were then incubated for 48 h in primary antibody to UCN1 (U4757; Sigma) or CRF (gift of Wylie Vale; Salk Institute, La Jolla, Calif., USA). These antibodies have been extensively characterized and previously applied to primate brain [37–39]. The UCN1 antibody was diluted 1/15,000 in 0.6% normal goat serum, 0.02 M KPBS and 0.4% Triton. The CRF antibody was diluted 1/3,000 in 0.1% human α -globulin-KPBS. The sections were then rinsed in KPBS buffer 4 times for 15 min each, incubated in Vector biotinylated goat anti-rabbit serum for 60 min, washed in KPBS buffer 4 times for 15 min each, incubated with Vector ABC reagent for 60 min, washed in KPBS buffer 4 times for 15 min each, incubated with 0.05% diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) containing 3% hydrogen peroxide for approximately 5 min, and finally washed in KPBS buffer 4 times for 15 min each. The sections were mounted on Superfrost Plus slides (Fischer, Santa Clara, Calif., USA) and dried overnight under vacuum. Then, they were further dehydrated via a graded series of ethanols, xylene and Histoclear. Finally, they were mounted under glass with DPX.

Stereological Analysis of Immunostaining

Midbrain sections were anatomically matched between animals using anatomical reference points. A Marianas Stereology Workstation (Intelligent Imaging Innovations, Denver, Colo., USA) with Slidebook 4.2 (Olympus Imaging Systems, Center Valley, Pa., USA) was used for analysis. Four levels of the dorsal raphe nucleus were immunostained for CRF fibers. Three levels of the midbrain, rostral to the dorsal raphe nucleus, were immunostained for UCN1 fibers. A montage of the entire area containing CRF- or UCN1-positive fibers was built by the workstation. The area containing the CRF- or UCN1-positive fibers was defined and measured (pixels and square micrometer total area). Next, the image was segmented into positive and negative pixels by Slidebook 4.2 and was examined at high magnification to verify that only specific-immunostained signal concentrated in fibers was highlighted. The positive pixel area was obtained and computed as percent of the total area in pixels.

As previously described by May et al. [39], the UCN1-positive cell bodies were located adjacent to the Edinger-Westphal nucleus in the supraoculomotor area (SOA), which is rostral to the UCN1 fiber area and the raphe nucleus. The area to be analyzed was outlined and a montage containing the SOA was obtained. The detectable UCN1-positive cells were marked by the observer and tallied by Slidebook 4.2. All cell counts refer to detectable neurons only. Cells were counted at 4 levels through the rostrocaudal extent of the SOA and the total number of UCN1-positive cells was obtained for each animal. The individual totals were averaged to obtain the group means. In addition, Slidebook 4.2 provides micrometer measures of the outlined area. The thickness of each section ($25\text{ }\mu\text{m} \times 4\text{ sections} = 100\text{ }\mu\text{m}$) was used as the length of the examined SOA region. The average area of the region for each animal (square micrometers outlined by Slidebook 4.2) was multiplied by the length to obtain the volume of the examined region (cubic micrometers/ 10^9 = cubic millimeters). The total number of UCN1-immunoreactive neurons in each animal was divided by the volume of the area examined to obtain the number of UCN1 neurons per cubic millimeter for each animal. The individual animal numbers of UCN1 neurons per cubic millimeter were then averaged to obtain the group means.

Statistical Analysis

The Student t test was applied for comparison of 2 groups. All data comparing 4 groups were subjected to two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc pairwise comparison. The variance within the groups reflects the difference between animals. Statistical analyses were conducted using the Prism Statistical software 5.0 (GraphPad Software Inc., San Diego, Calif., USA). $p < 0.05$ was considered statistically significant.

Results

CRF Fiber Staining

Robust CRF fiber staining was observed in the dorsal raphe nucleus. CRF fiber staining in the dorsal raphe nucleus from a representative animal in each group is illustrated in figure 1. The positively stained fibers were segmented and highlighted in blue. Perceptibly, there appears to be a decrease in CRF fiber density in the SS animal treated with S-citalopram (Cit) compared to the SS animal treated with Pb. The quantification of the CRF fiber density in Pb-treated animals at 4 levels of the dorsal raphe is shown in figure 2. The data are parsed into rostral (levels 1 and 2) and caudal (levels 3 and 4) regions of the dorsal raphe nucleus for the Pb-treated groups. There was a significant difference between HSR+Pb and SS+Pb groups in the caudal region of the dorsal raphe nucleus, with SS+Pb animals exhibiting a significantly higher CRF fiber density compared to HSR+Pb animals ($p = 0.0170$). There was no difference between the Pb-treated groups in the rostral region of the dorsal raphe nucleus. Figure 3 illustrates the effect of S-citalopram on overall CRF fiber density across all 4 levels of the dorsal raphe nucleus. Two-way ANOVA indicated that there was a significant effect of treatment ($p = 0.0496$), but no effect of stress sensitivity ($p = 0.6755$) and no interaction ($p = 0.3027$). There was a significant difference between the SS groups, with the SS+Cit group showing a significantly lower CRF fiber density compared to the SS+Pb group (Bonferroni's post hoc: $p < 0.05$). The HSR groups demonstrated no difference in CRF fiber density. There was no difference in the total area examined between the groups.

UCN1 Fiber Staining

Sections of the midbrain that were rostral to the dorsal raphe nucleus and encompassing the caudal linear nucleus contained a UCN1 fiber plexus that was used for analysis. UCN1 fiber staining in this area from representative HSR and SS animals is illustrated in figure 4. The posi-

Fig. 1. Photomicrographs of CRF fiber staining in the dorsal raphe nucleus. Stereology montage of CRF fiber staining in the dorsal raphe nucleus in a representative animal from each treatment group. Sections were immunostained for CRF and segmented into

positively and negatively stained pixels. Blue: positive pixels. Cell bodies or debris were erased prior to pixel quantitation. Visually, there appears to be a lower density of CRF fibers in the SS+Cit animal. $\times 10$.

Fig. 2. CRF fiber density expressed as percent of total pixel area covered by CRF-positive fibers (pixels) in the Pb-treated groups. Rostral and caudal regions of the dorsal raphe nucleus of the HSR and SS groups treated with placebo (HSR+Pb and SS+Pb, respectively). In the rostral region, there was no significant difference between the groups. In caudal region, there was a significant difference (F test: $p = 0.0170$) between HSR+Pb and SS+Pb groups, with the SS+Pb group exhibiting a significantly higher CRF fiber density compared to the HSR+Pb group.

Fig. 3. Average CRF-positive pixels across 4 levels of the dorsal raphe nucleus in all groups expressed as percent of total area examined. There was a significant effect of treatment ($p = 0.0496$), but no effect of stress sensitivity ($p = 0.6755$) and no interaction ($p = 0.3027$). The SS+Cit group exhibited a significantly lower CRF fiber density compared to the SS+Pb group. * Significant difference (Bonferroni: $p < 0.05$).

tively stained fibers were segmented and highlighted in blue. Any UCN1-positive neurons were eliminated before pixel counting. There appears to be a higher density of UCN1 fibers in the HSR animals compared to the SS animals. The quantification of the UCN1 fiber density across 3 levels of the midbrain is shown in figure 5. The average UCN1-positive pixel area as a percentage of the total area examined is shown. Two-way ANOVA indicated that there was a significant effect of stress sensitivity ($p = 0.047$), but no effect of S-citalopram treatment ($p = 0.495$) and no interaction ($p = 0.894$). Thus, there was a significant difference between the HSR and SS groups, with the HSR groups showing a significantly higher UCN1 fiber density compared to the SS groups, but S-citalopram had no effect (fig. 5). The total examined area did not differ between the groups.

UCN1 Cell Body Staining in SOA

The location of UCN1 cell bodies differs between rodents and primates. In our cynomolgus macaques, UCN1 cell bodies were located adjacent to the Edinger-Westphal nucleus in the supraoculomotor area (SOA) of the rostral midbrain. Our observations agree with and follow the anatomical characterization of UCN1 cell bodies in macaques by May et al. [39]. Figure 6 illustrates UCN1 cell body staining in this area from a representative animal. The quantification of the UCN1 cell bodies across

Fig. 4. Photomicrographs of UCN1-positive fibers in the midbrain, rostral to the dorsal raphe nucleus, in a representative HSR animal and an SS animal. Sections were immunostained for UCN1 and segmented into positively and negatively stained pixels. Blue: positive pixels. Cell bodies or debris were erased prior to pixel quantitation. Visually, there were few UCN1 fibers in the SS groups.

4 levels of the SOA is shown in figure 7. The number of neurons in the 4 levels was summed, generating the total number of neurons per animal. The average of the animal totals was then obtained for each group, and these data are shown in figure 7a. By two-way ANOVA, there was a significant effect of stress sensitivity ($p = 0.04$), but no effect of treatment ($p = 0.43$). There was a nearly significant interaction ($p = 0.056$), supporting the suggestion that S-citalopram increased detectable UCN1 neu-

Fig. 5. Average UCN1-positive pixels across 3 levels of the rostral midbrain in all groups expressed as percent of total area examined. There was a significant effect of stress sensitivity ($p = 0.0477$), but no effect of S-citalopram treatment ($p = 0.4958$) and no interaction ($p = 0.8946$). The SS groups exhibited a significantly lower UCN1 fiber density compared to the HSR groups. * Significant difference (ANOVA: $p < 0.05$).

Fig. 6. Photomicrograph of UCN1-positive cells in the SOA near the Edinger-Westphal nucleus (EW) in a representative animal. The cells are superior to the oculomotor (third) nucleus (III).

Fig. 7. Overall averages of UCN1-positive cells in the SOA. The number of neurons across 4 levels was summed, generating the total number of neurons per animal. Then, the average of the animal totals was obtained for each group. * Significant difference (ANOVA: $p < 0.05$). **a** Average number of UCN1-positive neurons. By two-way ANOVA, there was a significant effect of stress sensitivity ($p = 0.04$), but no effect of treatment ($p = 0.43$). There was a nearly significant interaction ($p = 0.056$), indicating that S-citalopram increased detectable UCN1 neurons in the SS group, but not in the HSR group.

opram increased detectable UCN1 neurons in the SS group, but not in the HSR group. **b** Average number of UCN1-positive neurons per cubic millimeter for each group. By two-way ANOVA, there was a significant effect of stress sensitivity ($p = 0.04$), but no effect of treatment ($p = 0.43$). There was a nearly significant interaction ($p = 0.056$), indicating that S-citalopram increased detectable UCN1 neurons in the SS group, but not in the HSR group.

rons in the SS group, but not the HSR group. In figure 7b, the number of UCN1-positive neurons per cubic millimeter volume for each group is illustrated. By two-way ANOVA, there was a significant effect of stress sensitivity ($p = 0.04$), but no effect of treatment ($p = 0.43$). There

was a nearly significant interaction ($p = 0.056$), further supporting the suggestion that S-citalopram increased the number of detectable UCN1 neurons in the SS group, but not in the HSR group. The volume examined did not differ between the groups.

Discussion

The neurobiological basis of individual differences in stress sensitivity and the potential pharmacological remediation are important issues in obvious fields such as psychiatry and immunology. More recently, gynecologists have recognized that individual differences in stress sensitivity play a role in the pathology of infertility [4, 40]. We have previously shown that monkeys who are sensitive to stress-induced amenorrhea exhibit significantly more CRF mRNA and CRF protein in the caudal region of the PVN, significantly more CRF mRNA in the thalamus and a significantly higher CRF fiber density in the central nucleus of the amygdala relative to stress-resilient animals under nonstressed basal conditions [13]. In contrast, rodent strains with different sensitivities to stress exhibit no difference in hypothalamic CRF expression [41].

In our model, stress induced reproductive dysfunction in sensitive individuals, but even in nonstressed conditions, SS monkeys have chronically lower ovarian steroid secretion. In a new cohort of animals, 15 weeks of S-citalopram treatment elevated estrogen and progesterone secretion in SS animals. Since S-citalopram is an SSRI, we have recently examined its effect on serotonin-related gene expression [9]. Although SS animals had a significantly lower expression of *Fev*, *TPH2*, *SERT* and *5-HT_{1A}* genes, S-citalopram had no effect. Nonetheless, the mechanism of action of S-citalopram on the serotonin system is well accepted. This led us to speculate that S-citalopram may elevate extracellular serotonin without affecting gene expression, and in turn, elevated extracellular serotonin may regulate other stress-related neural systems.

CRF-producing neurons and projections are widespread and play a role in stress-related psychiatric disorders [42]. CRF innervation of the serotonergic dorsal raphe nucleus has been described and CRF receptors are found in the dorsal raphe of rodents [23, 43, 44]. Moreover, CRF regulates serotonin release [28, 45–47].

Previous studies have also demonstrated that serotonin innervates CRF neurons in the hypothalamus [48, 49], and newer data indicate that serotonin decreases CRF [50, 51], perhaps via inhibitory interneurons [52]. Therefore, we hypothesized that S-citalopram increased extracellular serotonin, which decreased CRF production and delivery to extrahypothalamic regions including the dorsal raphe. This could further facilitate serotonin neurotransmission and further decrease CRF, thereby establishing a feed-forward loop. We also hypothesized that SS animals would exhibit a lower pro-

duction and delivery of UCN1, and that S-citalopram would increase UCN1.

This study found that, indeed, the CRF fiber density in the caudal portion of the dorsal raphe nucleus was increased in SS individuals, and that administration of S-citalopram reduced the CRF fiber density only in the SS group. The CRF innervation of the dorsal raphe is believed to originate in the caudal portion of the PVN and in the amygdala, where we had previously observed an increase in CRF in SS individuals relative to SR individuals [13]. This difference was reflected in the CRF fiber density in the dorsal raphe of the Pb-treated animals. Extrapolating from the current data, it is likely that S-citalopram reduces CRF gene and protein expression in the PVN and amygdala. Although a similar effect has been observed in rodents with other antidepressants [50, 51], the mechanism of action has not been addressed. We speculate that SSRIs elevate serotonin in the synaptic cleft, which in turn decreases CRF production and neurotransmission. Further studies to look at CRF in the hypothalamus and thalamus from this same cohort of animals are planned.

A small, but fairly consistent UCN1 fiber plexus amenable to measurement was detected in an area between the Edinger-Westphal nucleus and the dorsal raphe nucleus near the serotonergic caudal linear nucleus. As predicted, the UCN1 fiber density was significantly higher in HSR than in SS animals. However, S-citalopram had no effect on UCN1 fiber density although this little plexus may not represent UCN1 fiber density in other regions. Norepinephrine and the locus ceruleus have also been implicated in stress- and CRF-related peptide interactions [53], and cholinergic neurons in the Edinger-Westphal nucleus are adjacent to UCN1 neurons as well [39]. Clearly, the afferent innervation of the midbrain UCN1 neurons needs to be defined.

UCN1 decreases feeding behavior and also participates in the stress response [54–56]. UCN1 binds to CRF-R2 with greater affinity than CRF, suggesting that UCN1 preferentially binds to CRF-R2. Both UCN1 knockout mice and CRF-R2 knockout mice exhibit increased anxiety-like behavior [56]. Thus, it has been generalized that activation of CRF-R1 by CRF increases anxiety-like behavior, whereas activation of CRF-R2 by UCN1 decreases anxiety-like behavior. Our observations in HSR and SS animals are consistent with this generalization. SS animals have a higher CRF fiber density and lower UCN1 fiber density in the raphe region of the midbrain. In addition, we observed that SS animals exhibit more behavioral agitation, an indication of anxiety in primates (un-

published), as measured by undirected activity in novel settings of a free play test and the Human Intruder test [57]. It is reasonable to hypothesize that in SS animals, the higher CRF and lower UCN1 innervations lead to a decrease in serotonin neurotransmission, which in turn contributes to their stress sensitivity.

In rodents, the main population of urocortin-positive cells has been described as lying within the Edinger-Westphal nucleus of the midbrain. However, in primates, only a few urocortin cells are found within the confines of the Edinger-Westphal nucleus. The midbrain UCN1 cell bodies in macaques are located adjacent to the Edinger-Westphal nucleus, in an area referred to as the SOA [39]. These neurons also express cocaine and amphetamine regulated transcript or CART [25, 58]. There were more detectable UCN1-positive neurons in the SS groups than in the HSR groups, which is the inverse of the fiber density. It is attractive to speculate that the transport of UCN1 may be compromised in SS animals (which we also observed with GnRH) although other mechanisms cannot be excluded. However, it is important to note that the two-way difference between HSR and SS groups was largely due to the increase in detectable UCN1 neurons in the SS group treated with S-citalopram (SS+Cit). This was supported by the nearly significant difference in interaction, which indicates that S-citalopram had an effect in the SS group, but not in the HSR group. Indeed, the number of UCN1-positive neurons was similar in the HSR+Pb, HSR+Cit and SS+Pb groups. The basal, constitutive expression of UCN1 is moderate across species, but restraint stress rapidly elevates UCN1 mRNA in rodents [56]. None of the monkeys were stressed at the time of euthanasia. Hence, the modest and similar expression of UCN1 in the cell body region of the HSR+Pb, HSR+Cit and SS+Pb groups may be a reflection of environmental normality. The increase in detectable UCN1-positive neurons in the SS+Cit animals is consistent with the increase in UCN1 mRNA observed in rodents treated with the anti-anxiety drug alprazolam [59] although its mechanism of action differs from that of S-citalopram.

As noted above, S-citalopram treatment also increased peak estradiol and progesterone secretion in SS monkeys. This study and previous work [9] have approached the neural systems acted upon by S-citalopram, which in turn are thought to impinge upon the GnRH system and ultimately determine ovarian steroid secretion. However, it is also valid to question whether the increase in estrogen and progesterone in SS animals could cause the observed changes in the CRF and UCN1 systems. We have unpublished data showing that ovarian steroids decrease

CRF and increase UCN1 fiber density in the raphe region, thus we cannot rule out this possibility. However, ovarian hormones alter gene expression in serotonin neurons [60], and citalopram did not [9]. Perhaps it is the combination of elevated extracellular serotonin and ovarian steroids that influences the CRF and UCN1 systems in SS animals in a positive-feedback fashion.

In summary, in regions of the midbrain that contain serotonin cell bodies there is a higher density of CRF fibers and a lower density of UCN1 fibers in SS macaques compared to HSR macaques. In addition, with 15 weeks of S-citalopram treatment, there was a decrease in CRF fiber density in the dorsal raphe and a trend toward increased UCN1 production in the cell body region of SS animals. There was no effect of citalopram on UCN1 fiber density. The decrease in CRF delivered to the dorsal raphe could allow serotonin to further increase, producing a feed-forward circuit in which CRF was further decreased. Together, these actions could reduce anxiety and sensitivity to stress.

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