The Effect of Citalopram on Midbrain CRF Receptors 1 and 2 in a Primate Model of Stress-Induced Amenorrhea

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Abstract

We have demonstrated marked differences in the neurobiology of the serotonin system between stress-sensitive (SS) and stress-resilient (SR) cynomolgus macaques characterized in a model of stress-induced amenorrhea, also called functional hypothalamic amenorrhea (FHA). Dysfunction of the serotonin system in SS monkeys suggested that administration of a selective serotonin reuptake inhibitor (SSRI) might correct FHA. This study examines the effect of escitalopram (CIT) administration to SS and SR monkeys on corticotrophin-releasing factor (CRF) receptor 1 (CRF-R1) and CRF receptor 2 (CRF-R2) gene expression in the serotonin cell body region of the midbrain dorsal raphe. CRF-R1 was not significantly different between groups. There was a significant effect of treatment and a significant interaction between treatment and stress sensitivity on the average CRF-R2-positive pixel area ($P < .004$ and $P < .006$, respectively) and on the average number of CRF-R2-positive cells ($P < .023$ and $P < .025$, respectively). CIT significantly increased CRF-R2-positive pixel area and cell number in the SS group (pixel area $P < .001$; cell number $P < .01$; Bonferroni) but not in the SR group. In summary, CIT administration tended to decrease CRF-R1, but the small animal number precluded significance. CIT administration significantly increased CRF-R2 only in SS animals. These data suggest that the administration of CIT reduces anxiogenic components and increases anxiolytic components of the CRF system in the midbrain serotonin network, which in turn leads to improved ovarian function. Moreover, these data raise the possibility that SSRIs may be effective in the treatment of stress-induced infertility.

Keywords

stress, selective serotonin reuptake inhibitor, corticotropin-releasing hormone receptors, dorsal raphe, serotonin, and macaques

Introduction

A body of literature indicates that there is a link between stress and decreased reproductive function. About 30% of female amenorrhea is diagnosed as stress-induced amenorrhea or functional hypothalamic amenorrhea (FHA).¹ Functional hypothalamic amenorrhea is a disorder that occurs in females with no identifiable organic cause, who tend to score higher than average on psychometric tests for stress; who diet but do not qualify for anorexia or other eating disorders; who exercise regularly; and who have no menstrual cycles for over 6 months.²⁻⁵ Effective stress management results in restoration of fertility in most patients.^{6,7}

In order to study the underlying mechanism of FHA, we have developed an experimental nonhuman primate model in which mild psychosocial stress (relocation) combined with a metabolic stress (mild diet and/or moderate exercise regimen) leads to a suppression of reproductive function in some, but not all, animals. In our monkey model, we observe that female cynomolgus monkeys are either highly stress resilient (SR) and maintain normal menstrual cyclicity when exposed to combined stress across 2 menstrual cycles, or medium SR (MSR) with ovulation in the first stress cycle, but not in the

second stress cycle, or stress sensitive (SS) with cessation of ovulation as soon as stress is initiated.⁸ As in human patients, fertility is restored in nonhuman primates once the stress is removed. Also similar to women with FHA, SS animals have lower estrogen (E) and progesterone (P) secretion even in a nonstressed control environment.⁸

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There are 2 major central nervous systems (CNS) that govern stress responses, the CRF and serotonin systems.⁹ The paraventricular nucleus (PVN) of the hypothalamus contains CRF neurons involved in the stress response. Subpopulations of PVN neurons project to the midbrain raphe nuclei where CRF fibers contact serotonin neurons.^{10,11} CRF activates 2 known G-protein coupled receptors, CRF-R1 and CRF-R2,¹² and CRF has a higher affinity for CRF-R1 than CRF-R2.¹³ Both CRF-R1 and R2 are detected in the raphe nucleus and have opposing effects on serotonin release. Activation of CRF-R1 inhibits serotonergic activity and activation of CRF-R2 stimulates serotonergic activity.^{9,14} It is thought that CRF-R1 mediates anxious behavior and the hypothalamic–pituitary–adrenal (HPA) axis response to stress, while CRF-R2 mediates stress-coping behaviors such as anxiolytic behaviors, dearousal, and cardioprotection.¹⁵ Therefore, either inhibition of CRF-R1 and/or stimulation of CRF-R2 could decrease anxiety and related depressive behaviors. However, the relationship between reduced gonadotropin-releasing hormone (GnRH) drive, HPA activation, and anxiety has not been established.

A reciprocal relationship between the CRF and serotonin systems exists.⁹ The dorsal and median raphe nuclei send serotonergic projections to the forebrain and diencephalon, including the PVN.^{16,17} Our observations suggest that serotonin inhibits PVN-CRF production in primates,^{18,19} although other studies report serotonin stimulates CRF in rodents.²⁰ The dorsal raphe is a heterogeneous and bilateral nucleus located in the midbrain ventral to the periaqueductal grey. It contains approximately 235,000 neurons of which about 70% contain serotonin as the major neurotransmitter.²¹

Dysfunction of the CRF and serotonin systems is common in mood and anxiety disorders and they play critical a role in the stress response.^{22–25} In addition, the corticotropin-releasing hormone (CRH) and serotonin systems provide input into the hypothalamic–pituitary–gonadal axis and therefore may affect reproductive potential.^{26,27} We have previously demonstrated that there are pivotal differences between SS and SR animals in functional aspects of the serotonin and CRF systems. Stress-sensitive animals had lower release of serotonin after fenfluramine challenge,²⁸ and lower gene expression of *Fev*, *SERT*, *TPH2*, and *5-HT1A* receptor in the serotonergic dorsal raphe nucleus compared to SR animals.^{29,30} With regard to the CRF system, SS animals had higher *CRH* gene expression in the hypothalamic PVN and denser CRF fiber staining in the dorsal raphe nucleus than SR animals.^{19,31} The dysfunction of the serotonin system in SS monkeys suggested that administration of an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class might correct FHA.

Thus, we characterized a new population of monkeys as SS and SR. They were then administered the SSRI, Escitalopram (CIT), or placebo for 15 weeks in the absence of stress. Stress sensitive animals that were treated with CIT during nonstressed conditions exhibited a significant improvement in serum E and P concentrations, further supporting a connection between serotonin and regulation of the reproductive system.^{29,32} However, CIT had no effect on serotonin-related gene expression in

the same animal cohort.²⁹ We speculated that there might be other key elements that are affected by extracellular serotonin which, in turn, alter reproductive axis function. Indeed, CIT administration to SS animals decreased CRF fiber density and increased UCN1 fiber density in the midbrain raphe region compared to SS animals treated with placebo.³¹

In this study, we hypothesized that the expression of anxiogenic CRF-R1 receptors in the dorsal raphe would be higher in SS animals compared to SR animals; and that CIT administration may lessen this difference and decrease the expression of CRF-R1 receptors in SS macaques. We further hypothesized that the expression of anxiolytic CRF-R2 receptors in the dorsal raphe would be lower in SS animals compared to SR animals; and that CIT administration may increase the expression of CRF-R2 receptors in SS macaques. Thus, we characterized *CRF-R1* and *CRF-R2* gene expression in dorsal raphe of SS and SR animals after 15 weeks of CIT administration under non-stressed conditions.

Materials and Methods

All studies were reviewed and approved by the Institutional Animal Care and Use Committee of ONPRC and performed according to federal guidelines.

Animal care, animal sensitivity characterization, the CIT treatment paradigm, determination of therapeutic serum concentrations of CIT, determination of serum concentrations of steroid hormones, and animal selection were previously described in detail.^{29,31,32} After sensitivity characterization, SR and SS animals were returned to nonstress conditions and treated with placebo or a therapeutic concentration of CIT (1.2–4.8 mg/kg) for 15 weeks. The dose administered was individually adjusted to obtain a therapeutic dose between 20 and 40 ng/mL. Three weeks after a final adjustment, blood levels of s-citalopram at 3 and 6 hours 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. The animals were euthanized after 15 weeks of treatment.^{29,31,32}

In SS monkeys, citalopram treatment significantly increased peak estradiol levels in the follicular phase of the menstrual cycle from 360 ± 67 to 544 ± 82 pg/mL ($P < .05$) and increased peak P levels in the luteal phase of the menstrual cycle from 6.7 ± 1.4 to 11.3 ± 1.8 ng/mL ($P < .05$). In contrast, vehicle- and citalopram-treated SR monkeys had similar peak estradiol and peak P, before and after treatment, which averaged (both groups) 575 ± 100 pg/mL and 11.5 ± 3 ng/mL, respectively.^{29,31,32}

The monkeys were euthanized according to the procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Perfusion and preparation of the brain, sectioning, and storage of sections were previously described in detail.²⁹ For neuroanatomical analysis, we used a 2×2 block design with 4 animals in each group, designated SR + placebo, SR + citalopram, SS + placebo, and SS + citalopram.

Complete coding sequences for macaque CRF-R1 and CRF-R2 were obtained from PubMed. Unique forward and reverse

primers were constructed (Invitrogen Life Technologies, Carlsbad, California) using Primer Express Software. The primers were as follows:

CRF-R1 Forward: TTTTCAACATCGTCCGCATC
Reverse: ATGGCAGAACGGACCTCACT
CRF-R2 Forward: CACAGTGTGAGCCCATTTTGG
Reverse: TTCGCAGGATAAAGGTGGTGA

The primers were used in a polymerase chain reaction (PCR) against reverse transcribed RNA (complementary DNA [cDNA]) from a small block of monkey midbrain containing the dorsal raphe nucleus. The cold PCR included 25 ng cDNA, 2.5 mmol/L dNTPs, 1.5 mmol/L MgCl₂, 1× PCR buffer (Invitrogen), 10 nmol/L forward and reverse primers and 0.5 Unit of platinum Taq polymerase (Invitrogen). The amplicons were sliced from a 1% agarose gel, purified with Qiaquick PCR purification kit (Qiagen, Germantown, Maryland) and subcloned into pGEM-T vector. The CRF-R1 primers amplified 262 base pairs of the CRF-R1 coding region corresponding to bp 1086 to 1347. The CRF-R2 primers amplified 200 base pairs of the CRF-R1 coding region corresponding to bp 353 to 553. The direction of the insert in the vector was determined by sequencing the insert. Each insert was transcribed in the antisense direction with T7 and in the sense direction with SP6.

The expression of CRF-R1 and CRF-R2 was determined with a digoxigenin in-situ hybridization (ISH) assay as previously described.^{29,33} Images of the dorsal raphe were captured with a Marianas Stereological Workstation. Cell number and positive pixel area were obtained with ImageJ to quantify the ISH-digoxigenin signal.^{29,31} All measurements were averaged across the levels, generating one overall average for each animal. In addition, the measurements were totaled for all levels generating one overall total for each animal. The data were compared with a 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc pairwise comparison. Thus, the variance reflects the difference between animals. In all analyses, the outlined area subjected to analysis was held constant for each level. All statistical analyses were conducted using the Prism Statistic Program 5.0 (GraphPad, San Diego, California). A confidence level of $P < .05$ was considered significant.

Results

Riboprobes and Macaque CRF Receptor cDNA Clones

The specific primers produced single amplicons from a reverse transcribed extraction of a monkey dorsal raphe, which were then inserted into pGEM-T and sequenced. The sequences were identical to the predicted sequence from PubMed. The sequences of each subclone were entered into Sequencer 4.10 software and compared. There was no alignment between 60% and 90% stringency. This indicates that under the conditions of our assay, neither of the specific riboprobes would bind to RNA corresponding to the other receptor.

Corticotrophin-Releasing Factor Receptor 1

Corticotrophin-Releasing Factor R1 messenger RNA (mRNA) was robustly expressed in the dorsal raphe nuclei, the raphe interfascicularis, and the median raphe. Figure 1 illustrates the CRF-R1 digoxigenin-ISH staining observed in the dorsal raphe of a SS animal treated with placebo at different magnifications (panels A-C). The negative control is also illustrated in panel D, in which a section was hybridized with a sense riboprobe. The staining was obliterated with minor nonspecific staining remaining. The nonspecific staining was identical to that of sections processed in the absence of probe, or sections preincubated with RNase and then hybridized to sense probe. Numerous cells were positive for CRF-R1, but staining intensity varied between cells. Image analysis of the CRF-R1 signal included quantitation of the CRF-R1-positive pixel area and number of CRF-R1-positive cells in each section/level. First, the average of each parameter was obtained across 4 levels of the dorsal raphe and then the average of the animals in each group was obtained (Table 1). The differences between the groups in the average positive pixel area and cell number were not statistically different although a trend was observed. The difference between the SS groups and the SR groups in the average pixel area did not reach statistical significance ($P = .08$). The SS groups tended toward higher average CRF-R1-positive pixel area and more CRF-R1-positive cells than the SR groups.

In addition, the total of each parameter was obtained for each animal by adding all of the 4 levels examined and then the average of the animal totals was obtained for each group. There was a trend toward a reduction in both CRF-R1 total positive pixel area and positive cell number when the SS animals were treated with CIT (Table 2). The difference between placebo and treatment groups in the total cell number did not reach statistical significance ($P = .08$). The CIT-treated groups tended to have fewer total numbers of CRF-R1-positive cells; and the SS + CIT group trended toward a reduction in total positive pixel area compared to the SS + placebo group.

Corticotrophin-Releasing Factor Receptor 2

Corticotrophin-Releasing Factor R2 mRNA was robustly expressed in the dorsal raphe nucleus, the raphe interfascicularis, and the median raphe. Figure 2 illustrates the CRF-R1 digoxigenin-ISH staining observed in the dorsal raphe and raphe interfascicularis of a SR animal treated with placebo. The negative control is also illustrated in panel C, in which a section was hybridized with a sense riboprobe. The staining was nearly obliterated with minor nonspecific staining remaining. The nonspecific staining was identical to that of sections processed in the absence of probe, or sections preincubated with RNase and then hybridized to sense probe. There was no difference in the nonspecific binding between CRF-R1 and CRF-R2 assays. Numerous cells were positive for CRF-R2 but the intensity varied between cells.

Figure 3 contains low-magnification images of the CRF-R2 digoxigenin-ISH staining observed in the dorsal raphe of a

Figure 1. Illustration of the digoxigenin in-situ hybridization (ISH) signal for CRF-R1 expression in the dorsal raphe of a stress-sensitive monkey treated with placebo at different magnifications (panels A-C) and a section processed with sense CRF-R1 probe (D). CRF-R1 indicates corticotrophin-releasing factor receptor 1.

Table 1. Overall Average Number of CRF-R1-Positive Pixel Area and Average CRF-R1-Positive Cells Across 4 Levels of the Dorsal Raphe

Average CRF-R1-Positive Pixel Area		
Sensitivity ^a	Placebo	Citalopram
SR	22 510	24 390
SS	31 140	28 660
Average CRF-R1-Positive Cells		
Sensitivity	Placebo	Citalopram
SR	609.8	538.1
SS	667.8	598.1

Abbreviations: ANOVA, analysis of variance; CRF-R1, corticotropin-releasing hormone receptor 1; SS, stress sensitive; SR, stress resilient.
^a $P < .08$, ANOVA.

Table 2. Total CRF-R1-Positive Pixel Area and Total Number of CRF-R1-Positive Cells in 4 Levels of the Dorsal Raphe

Average Total CRF-R1-Positive Pixel Area		
Sensitivity	Placebo	Citalopram
SR	90 030	99 670
SS	122 500	114 600
Average Total CRF-R1-Positive Cells		
Sensitivity	Placebo	Citalopram ^a
SR	2439	2006
SS	2644	2392

Abbreviations: ANOVA, analysis of variance; CRF-R1, corticotropin-releasing hormone receptor 1; SS, stress sensitive; SR, stress resilient.
^a $P < .08$, ANOVA.

Figure 2. Illustration of the digoxigenin in-situ hybridization (ISH) signal for CRF-R2 expression in the dorsal raphe of a stress-resistant monkey treated with placebo at different magnifications (panels A, B) and a section processed with sense CRF-R2 probe (C). CRF-R2 indicates corticotrophin-releasing factor receptor 2.

representative animal from each treatment group. There appears to be fewer positive cells and lower signal intensity in the SS + placebo animal than in the SR + placebo animal. In addition, there appears to be an increase in CRF-R2-positive cells and signal intensity in the SS animal treated with CIT.

Image analysis of the CRF-R2 signal included quantitation of the CRF-R2-positive pixel area and number of CRF-R2-positive cells. The average of each parameter was obtained across 5 levels of the dorsal raphe; and then the average of the animals in each group was obtained (Figure 4). There was a significant difference in the CRF-R2 average positive pixel area between the groups due to treatment ($P < .004$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .006$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .001$; Bonferroni posttest). There was also a significant difference in the CRF-R2 average cell number between the groups due to treatment ($P < .02$), and there was a significant difference due to interaction of sensitivity and treatment ($P < .03$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($p < .01$; Bonferroni posttest).

In addition, the total of each parameter was obtained for each animal by adding all of the 5 levels examined and then the average of the animal totals was obtained for each group (Figure 5). There was a significant difference in the CRF-R2 total positive pixel area between the groups due to treatment ($P < .009$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .005$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .01$; Bonferroni posttest). There was also a near significant difference in the CRF-R2 total cell number between the groups due to treatment ($P < .06$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .03$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .05$; Bonferroni posttest).

Discussion

In clinical populations, as well as in most animal species studied to date, there is abundant evidence that severe stress can suppress the activity of the hypothalamic–pituitary–gonadal axis and thereby lead to a state of infertility. Severe stress can range from metabolic (famine) to psychosocial (war). For any stress, the primary locus of dysfunction in the reproductive axis appears to be at the level of the CNS, leading to a decrease in GnRH stimulation of the pituitary, a decrease in pituitary release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and eventually to a decrease in the activity of the gonads, resulting in anovulation and amenorrhea in females.^{34,35}

One of the most common forms of infertility is stress-induced infertility, often called functional hypothalamic amenorrhea

Figure 3. Low-resolution pictures from representative animals showing *CRF-R2* gene expression detected with digoxigenin-ISH. The animals from all 4 experimental groups: SS + P, SR + Cit, SR + P, and SR + Cit are compared. Note that *CRF-R2* gene expression in SS + P animal is much lower compared to the placebo-treated animals in SR group. Also, SS animals that were treated with Citalopram demonstrated higher *CRF-R2* gene expression compared to SS animals in the placebo group. *CRF-R2* indicates corticotrophin-releasing factor receptor 2; SS, stress sensitive; SR, stress resilient; CIT, escitalopram; P, progesterone.

(FHA).³⁶ Functional hypothalamic amenorrhea appears to result from exposure to a combination of mild or common psychosocial and metabolic life stresses. Clearly, not all women who experience these everyday life stresses develop fertility problems, suggesting that there is a range of sensitivity to developing stress-induced reproductive dysfunction in normal women.

In order to study the underlying pathophysiology of FHA, we developed an animal model in which monkeys are exposed to mild psychosocial stress (relocation) combined with metabolic stress (mild diet and/or moderate exercise regimen) for 2 months. This causes complete suppression of ovulation in about one third

of the animals, which we call stress sensitive (SS). Another one third of the animals continue to ovulate, and they are called stress resilient (SR). The remaining animals show intermediate responses. Better stress management strategies or completion of CBT aids in restoration of fertility in human patients with FHA.³⁴ Similarly, nonhuman primates resume ovulation once stress is removed.²⁸ Stress removal is plausible in experimental animal models, but unfortunately not usually achievable in humans, where stress-coping techniques fulfill this role.

In our primate model, SS animals show significant alterations in functions of the serotonin system. Compared to SR

Figure 4. The average CRF-R2 positive pixel area and positive cell number in the raphe nucleus across 5 morphological levels. **Top.** There was a significant difference in the CRF-R2 average positive pixel area between the groups due to treatment ($P < .004$) and there was a significant difference due to the interaction of sensitivity and treatment ($P < .006$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .001$; Bonferroni posttest). **Bottom.** There was also a significant difference in the CRF-R2 average cell number between the groups due to treatment ($P < .02$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .03$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .01$; Bonferroni posttest). CRF-R2 indicates corticotrophin-releasing factor receptor 2; SS, stress sensitive; CIT, escitalopram.

animals, SS animals had lower release of serotonin after fenfluramine challenge²⁸ and lower serotonin gene expression such as decreased *Fev*, *TPH2*, *SERT*, and *5HT1A* receptors in the serotonin neurons of the dorsal raphe.^{29,30} These observations suggested that a selective serotonin uptake inhibitor might alleviate the apparent reduction in serotonin function in SS animals.

Therefore, we characterized a new population of monkeys as SS and SR and then treated them with Escitalopram (CIT) or placebo for 15 weeks in the absence of stress.³² CIT selectively inhibits serotonin reuptake in the presynaptic neurons

Figure 5. The total CRF-R2-positive pixel area and positive cell number in the raphe nucleus across 5 morphological levels. **Top.** There was a significant difference in the CRF-R2 total positive pixel area between the groups due to treatment ($P < .009$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .005$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .01$; Bonferroni posttest). **Bottom.** There was also a near significant difference in the CRF-R2 total cell number between the groups due to treatment ($P < .06$) and there was a significant difference due to interaction of sensitivity and treatment ($P < .03$), but there was no difference due to sensitivity. The SS + CIT group was significantly higher than the SS + placebo group ($P < .05$; Bonferroni posttest). CRF-R2 indicates corticotrophin-releasing factor receptor 2; SS, stress sensitive; CIT, escitalopram.

in the brain thus elevating synaptic serotonin. It is used clinically to treat depression and anxiety, and it displays almost no affinity for serotonin, dopamine, adrenergic, or γ -aminobutyric acid (GABA) receptor subtypes.³⁷ In humans, the onset of action can manifest from 1 to 6 weeks after the initiation of drug therapy.^{38,39}

In our model, SS animals that were treated with CIT during nonstressed conditions demonstrate statistically significant improvement in serum estradiol and P concentrations, suggesting that there is a connection between serotonin and regulation of the reproductive system. However, the same animal cohort failed to demonstrate any changes in serotonin gene expression after CIT treatment, implying that there are other key elements

that are affected by extracellular serotonin, which can alter reproductive function.²⁹

In addition to serotonin, the CRF system plays an important role in stress responses. Corticotrophin-releasing factor exerts its effects on the serotonin system via 2 distinct receptors, the CRF-R1 and CRF-R2.^{9,14} In rodents, activation of CRF-R1 and CRF-R2 may cause opposite effects in terms of serotonin regulation with CRF-R1-inhibiting serotonin function and CRF-R2-stimulating serotonin function, but this may depend on the concentration of CRF reaching the receptors as well as the timing of exposure in various cellular preparations.^{40,41} In our primate model of FHA, we have found that SS monkeys have higher CRF fiber density, hence greater CRF-R1 activation; and lower UCN1 fiber density, thus lower CRF-R2 activation, in the raphe region when compared to SR monkeys. Moreover, CIT reduced CRF fiber density in the dorsal raphe.³¹

Corticotrophin-releasing factor R1 is loosely described as an anxiogenic receptor. Studies with CRF-R1 knockout mice and CRF overexpressing rodents revealed that anxiety and depression prone phenotypes are directly linked to the activation of CRF-R1 receptors in limbic structures.⁴² A recent double-blind placebo-controlled study revealed that a specific CRF-R1 antagonist reduced anxiety-related symptoms in a human experimental anxiety model.⁴³ Corticotrophin-releasing factor R2 is thought to be involved in mediating anxiolytic functions of CRF. Higher levels of CRF, or urocortins, are needed to activate CRF-R2, which increases serotonergic activity in the dorsal raphe.¹⁴ Mice lacking CRF-R2 tends to have anxiety prone phenotypes and lower stress-coping abilities.¹⁵ Central administration of CRF-R2 antagonists also reduces the ability to cope with stress in animals.¹⁵

In our model of FHA, SS animals appear to have higher anxiety and poor stress-coping mechanisms. Therefore, we hypothesized that SS animals would have higher expression of anxiogenic CRF-R1 in the serotonergic dorsal raphe nucleus compared to the SR animals, and that CIT treatment may eliminate this difference. We found that SS animals had a trend toward higher expression of CRF-R1 in the dorsal raphe nucleus compared to SR monkeys, but this result did not reach statistical significance. Also, SS animals that were treated with CIT demonstrated a trend toward a reduction in CRF-R1 expression compared to SS individuals treated with placebo. The lack of statistical significance may be derived from the small number of animals in each group. For example, in a previous experiment, we did not detect a statistical difference in *5HT1A* expression, but with more animals the difference was obvious.^{29,30} Thus, we run the risk of making a type II error wherein a real difference is not detected with a small number of monkeys due to budget constraints.

There is a polymorphism in the *CRF-R1* gene in humans. Homozygosity of GAG haplotype in CRF-R1 was associated with greater response to fluoxetine,⁴⁴ but this has not been examined in nonhuman primates. Further studies are needed to identify polymorphism in monkeys to better understand the regulation of the different components of the CRF system in response to pharmacotherapies.

The SR animals demonstrate less anxiety as demonstrated by reduced agitation, which may indicate a tendency toward better stress coping behaviors.⁴⁵ Therefore, we hypothesized that the expression of anxiolytic CRF-R2 receptors in the dorsal raphe would be higher in SR animals compared to SS animals, and that CIT administration may increase the expression of CRF-R2 in SS macaques. We found that CIT did in fact increase *CRF-R2* gene expression in the dorsal raphe of only the SS animals, although the difference between SR and SS placebo-treated animals was not significant. The analysis indicating a significant interaction suggests that CIT interacted with the stress-sensitivity status of the animals and elicited a response only in the SS group. This observation provides further insight into the underlying pathophysiology of FHA and suggests that CIT administration may alleviate aspects of the CRF receptor pathophysiology.

At this point, the mechanism by which CIT increases CRF-R2, and perhaps decreases CRF-R1, remains unknown. At this time we can only speculate about the underlying mechanism. It could be a direct action whereby CIT increased extracellular serotonin in the vicinity of the neuronal cell body and serotonin feedback on the serotonin cell body somehow altered expression of both receptors. It could also be an indirect action whereby CIT increased extracellular serotonin leading to elevated sex steroid concentrations, which in turn caused an increase in CRF-R2 expression, again in an unknown fashion. Rodent studies suggest that CRF-R1 is located on GABA neurons that inhibit serotonin, whereas CRF-R2 is located on, and directly stimulates, serotonin neurons.⁴⁶ In rats, *CRF-R1* gene expression was very low in the raphe, but it colocalized with serotonin and nonserotonin neurons. Expression of CRF-R2 was robust in the rat raphe, and it colocalized entirely with serotonin neurons at rostral levels, and with serotonin and GABA neurons at caudal levels.⁴⁷ Unlike rodents, CRF-R1 expression is robust in the monkey raphe and it mirrors CRF-R2 expression. If both receptors are in serotonin neurons, then studies of the promoters of each gene may reveal the divergent regulatory mechanism of the expression patterns. Future studies with double ISH to definitively characterize the phenotype of the neurons expressing R1 and R2 in monkey are intended.

We have previously observed that SS animals, like humans with FHA, have decreased LH pulse frequency, which then leads to decreased levels of ovarian steroid hormone levels.^{48–50} We previously showed that CIT treatment elevated E and P in SS animals.⁵¹ Hence, it is attractive to speculate that an increase in CRF-R2 expression in the dorsal raphe would increase serotonin neurotransmission, thereby increasing distant serotonin release in other brain structures and “fixing” the defect in FHA.

In conclusion, the dorsal raphe nucleus contains serotonin neurons that respond to CRF and that send projections to the forebrain limbic circuits. The serotonergic innervation of the central nucleus of the amygdala, hippocampus, and hypothalamus are involved in regulating behaviors like anxiety and fear.²¹ The interactions of the CRF and serotonin system are emerging as an important link in FHA pathophysiology. Our

results uncover yet another dysfunction in the CRH system in the FHA model that appears to be reversed by SSRI therapy.

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