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The effect of short-term stress on serotonin gene expression in high and low resilient macaques

Cynthia L. Bethea [a,b,c,d,*], Kenny Phu [a], Arubala P. Reddy [a], Judy L. Cameron [e]

[a] Division of Reproductive Sciences, Oregon National Primate Research Center, Beaverton, OR 97006, United States
[b] Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR 97006, United States
[c] Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97201, United States
[d] Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, OR 97201, United States
[e] Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA 15213, United States

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ABSTRACT

Female cynomolgus monkeys exhibit different degrees of reproductive dysfunction with moderate metabolic and psychosocial stress. When stressed with a paradigm of relocation and diet for 60 days, or 2 menstrual cycles, highly stress resilient monkeys continue to ovulate during both stress cycles (HSR); medium stress resilient monkeys ovulate once (MSR) and stress sensitive monkeys do not ovulate for the entire 60 days (SS). This study examines serotonin-related gene expression in monkeys with different sensitivity to stress and exposed to 5 days of moderate stress. Monkeys were first characterized as HSR, MSR or SS. After resumption of menstrual cycles, each monkey was re-stressed for 5 days in the early follicular phase. The expression of 3 genes pivotal to serotonin neural function was assessed in the 3 groups of monkeys ($n = 4–5$/group). Tryptophan hydroxylase 2 (TPH2), the serotonin reuptake transporter (SERT), and the 5HT1A autoreceptor mRNAs expression were determined at 4 morphological levels of the dorsal raphe nucleus with in situ hybridization (ISH) using digoxigenin-incorporated riboprobes. In addition, cFos was examined with immunohistochemistry. Positive pixel area and/or cell number were measured. All data were analyzed with ANOVA (3 groups) and with a *t*-test (2 groups). After 5 days of stress, TPH2, SERT, 5HT1A and cFos were significantly lower in the SS group than the HSR group ($p<0.05$, all). This pattern of expression was the same as the pattern observed in the absence of stress in previous studies. Therefore, the ratio of the HSR/SS expression of each serotonergic gene was calculated in the presence and absence of stress. There was little or no difference in the ratio of HSR/SS gene expression in the presence or absence of stress. Moreover, cFos expression indicates that overall, cell activation in the dorsal raphe nucleus and periaquaductal gray is lower in SS than HSR animals. These data suggest that the serotonin system may set the sensitivity or resilience of the individual, but serotonin-related gene expression may not rapidly respond to moderate stress in nonhuman primates.

1. Introduction

1.1. Functional hypothalamic amenorrhea

Exposure to stressful stimuli can lead to a variety of secondary diseases such as anxiety, depression, cardiovascular disease, and immune hyperactivity (Golovatscka et al., 2012; Priyadarshini and Aich, 2012). Reproductive dysfunction has been recently added to this growing list of stress-related disorders (Cameron, 2000; Xiao et al., 1999). It is now apparent that some of the neuroendocrine abnormalities associated with functional hypothalamic amenorrhea (FHA) are indicative of metabolic stress (Berga and Girton, 1989; Berga et al., 1997; Biller et al., 1990; Giles and Berga, 1993; Kondoh et al., 2001; Laughlin et al., 1998; Meczekalski et al., 2000), and that there is a high incidence of eating abnormalities in this patient population (Perkins et al., 2001; Warren and Fried, 2001; Warren and Stiehl, 1999). Moreover, treatment therapies for FHA that target both strategies for coping with psychological

Abbreviations: HSR, highly stress resilient; MSR, medium stress resilient; SS, stress sensitive; TPH2, tryptophan hydroxylase 2; SERT, serotonin reuptake transporter; 5HT1A, serotonin 1A receptor; ISH, in situ hybridization; IHC, immunohistochemistry; ANOVA, analysis of variance; cFos, immediate early gene; FHA, Functional Hypothalamic Amenorrhea; E, estradiol; P, progesterone; mRNA, messenger RNA; Fev, primate serotonin master gene (Pet1); CRF-R1, corticotropin releasing factor receptor 1; CRF-R2, corticotropin releasing factor receptor 2; KPBS, potassium phosphate buffered saline; DMSO, dimethyl sulfoxide; cDNA, copy DNA; cRNA, copy RNA; pGEMT, type of plasmid vector; SAC I, restriction enzyme; SAC II, restriction enzyme; SP6, specific transcription start site; T7, specific transcription start site; UTP, uridine triphosphate; DTT, dithiothreonine; DAB, diaminobenzadine; IgG, immunoglobulin G; PAG, periaquaductal gray; DEPC, RNase inhibitor for water treatment.

* Corresponding author at: Oregon National Primate Research Center, 5005 NW 185th Ave, Beaverton, OR 97006, United States. Tel.: +1 503 690 5327; fax: +1 503 690 5384.
E-mail address: betheac@ohsu.edu (C.L. Bethea).

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stress and removal of metabolic stresses look very promising (Berga et al., 2003). However, it is also clear that some individuals are very sensitive to stressors, while others are stress resilient.

We have developed an experimental nonhuman primate model of hypothalamic amenorrhea in which mild psychosocial stress combined with a mild diet, plus or minus a moderate exercise regimen, lead to a suppression of reproductive function that reverses upon stress removal (Bethea et al., 2008; Cameron, 2000; Williams et al., 1997). Female cynomolgus monkeys are either [1] highly stress-resilient (HSR) and maintain normal menstrual cyclicity when exposed to two cycles of combined stress, or [2] medium stress-resilient (MSR) and ovulate in the first stress cycle, but not in the second stress cycle, or [3] stress-sensitive (SS) and become anovulatory as soon as stress is initiated (Bethea et al., 2008; Cameron, 2000; Williams et al., 1997). Stress-sensitive individuals also have higher basal heart rates throughout the 24 h day compared to more stress-resistant animals (Cameron et al., 1998).

1.2. Serotonin and stress

The serotonin neural system plays a pivotal role in numerous autonomic functions in response to stress, as well as mood and affective regulation, cognition and satiety (Azmitia and Gannon, 1986; Jacobs and Azmitia, 1992; Mann et al., 1996; Van de Kar, 1991). Decreased activity of the central serotonin system is found in individuals with increased stress sensitivity and anxiety disorders (Bhagwagar et al., 2002; Tancer et al., 1994). In addition, stress impacts serotonin function in a variety of ways depending on the intensity and duration of the stress (Botchin et al., 1994; Filipenko et al., 2002; Shively et al., 1995). Serotonin neurotransmission is generally thought of as a combination of synthesis, release, turnover, neural activity and degradation. Pivotal proteins governing these functions are translated from mRNAs coding tryptophan hydroxylase (TPH), the serotonin reuptake transporter (SERT) and the 5HT1A autoreceptor.

In previous studies, the stress-sensitivity of each animal was determined with a 5-month protocol (Bethea et al., 2008), and then the animals were allowed to recover. After resumption of menstrual cycles, HSR animals released significantly more prolactin than SS animals in response to fenfluramine (Bethea et al., 2005a). This observation suggested there was an endogenous difference in the function of the central serotonergic system in SS versus HSR animals in the absence of stress. Each animal was then euthanized on day 5 of a non-stressed menstrual cycle. In the midbrain of these animals, we found that SS animals have lower expression of TPH2, SERT and 5HT1A mRNAs, as well as Fev, the serotonin master gene, in the dorsal raphe compared to HSR animals. SS animals also have higher CRF expression, greater CRF innervation of the dorsal raphe and lower expression of CRF-R2 receptors in the dorsal raphe compared to HSR animals (Bethea et al., 2008).

It is important to remember that in the absence of stress, SS and HSR animals ovulate normally and no observable difference is discernible. However, SS animals have a lower pre-ovulatory surge of estradiol (E) than HSR animals. This results in poor corpus luteum formation by the ovulatory follicle and lower production of progesterone (P). The administration of the selective serotonin reuptake inhibitor, citalopram, significantly increased E and P secretion in SS animals up to the levels secreted by the HSR animals, but HSR animals did not change with citalopram (Bethea et al., 2011; Cameron et al., 2004; Lima et al., 2009). Hence, the observations (1) that SS monkeys have lower release of serotonin/prolactin following fenfluramine administration, (2) that SS monkeys have lower expression of TPH2, SERT and 5HT1A (3) that citalopram increased ovarian steroid production only in SS animals, and (4) a body of literature that has shown various serotonergic responses to stress across species, altogether strongly implicated the serotonin system in the mediation of stress and stress sensitivity. Investigation of 3 genes that code for pivotal regulatory proteins in the serotonin system after short-term stress follows an obvious line of reasoning from the previous observations.

With this study, we begin to examine aspects of neurobiology in HSR and SS animals in the presence of stress. Initially, we questioned what effect short-term stress had on serotonin-related gene expression. Our hypothesis was that HSR animals would cope with stress and exhibit a stress-response in the serotonin system with an increase in serotonin-related gene expression, whereas SS animals would not. Surprisingly, this hypothesis was null. We present data indicating that the serotonin system continued to reflect stress sensitivity, but it was not markedly affected by 5 days of moderate stress.

2. Methods and materials

2.1. Animals and treatments

This experiment was approved by the IACUC of the Oregon National Primate Research Center and conducted in accordance with the 2011 Eight Edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Fifteen adult female cynomolgus monkeys (*Macaca fascicularis*) were utilized. The animals were 7–9 years of age with no prior pregnancies. The animals were imported from the and immediately housed in single cages in the same room. Cynomolgus macaques form social hierarchies in grouped housing with subordinates receiving more aggression. With single cages, the rank is not a variable. No information is available regarding their social status prior to import. They are the same group of animals utilized in previous publications examining activity of the HPA axis in response to mild psychosocial and metabolic stress (Herod et al., 2011a) and to a CRF-R1 antagonist (Herod et al., 2011b). The monkeys were housed at the Oregon National Primate Research Center (ONPRC) in individual stainless steel cages (32×24×27 in.) in a temperature-controlled room (23±2 °C) with lights on for 12 h/day (0700–1900). Animals were fed two meals a day consisting of six high-protein monkey chow biscuits (no. 5047, jumbo biscuits; Ralston Purina, St. Louis, MO) at 0930 and 1530, and a supplement of one-quarter piece of fresh fruit was provided with the afternoon meal. Animals had their vaginal area swabbed daily to check for menses. The first day of menses was designated as day 1 of a menstrual cycle. Food intake, measured just before the next meal was fed, was recorded for each meal, and weight was measured weekly. The monkeys were similar in weight, and there were no body weight changes throughout the characterization of stress sensitivity (Herod et al., 2011a).

2.2. Assessment of stress sensitivity

For each monkey, sensitivity of the reproductive axis to stress was categorized by assessing changes in menstrual cycle length, ovulation, and reproductive hormone secretion when monkeys were exposed to a mild psychosocial and metabolic stressor, as described previously (Bethea et al., 2008). This study was performed after each monkey had been living in its home cage surrounded by familiar monkeys for several months. To provide a standardized mild psychosocial stress, monkeys were moved on the first day of their menstrual cycle from their home cage to a single cage in a novel room, surrounded by unfamiliar monkeys. As a metabolic stress, each animal's available caloric intake was reduced by 20%. Blood samples (0.6 ml/sample) were taken every other day to assess reproductive steroid hormone concentrations using the blood collection protocol previously described. Monkeys that menstruated within 38 days subsequent to the initiation of stress were moved for a second stress cycle and remained on 20% lower calorie intake (Williams et al., 1997; Williams et al., 2001). Monkeys that did not mense were not moved a second time.

Animals were categorized as HSR if they presented a normal ovulatory menstrual cycle [25–38 days in length, peak E_2 > 200 pg/ml in follicular phase, peak P_4 > 2 ng/ml in luteal phase] in stress cycle 1