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The Effect of Short-term Stress on Serotonin Gene Expression in High and Low Resilient Macaques

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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 periaqueductal 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.

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Keywords

Stress; stress resilient; ovulation; amenorrhea; serotonin; macaques; gene expression

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 et al., 1997, Berga and Girton, 1989, 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 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, Centeno, 2008, Cameron, 2000, Williams, Berga, 1997). Stress-sensitive individuals also have higher basal heart rates throughout the 24 hr 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, Centeno, 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, Centeno, 2008).

It is important to remember that in the absence of stress, SS and HSR animals ovulate normally and no observable difference is discernable. 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.

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, Dettmer, 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, Centeno, 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, Berga, 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 and again in stress cycle 2. MSR animals were defined as those animals that presented a normal ovulatory menstrual cycle in response to stress cycle 1 but failed to mense by day 38 of stress cycle 2. Animals that immediately suppressed normal menstrual cyclicity upon exposure to stress (i.e they failed to ovulate and mense within 60 days) were categorized as SS. Animals that exhibited disrupted menstrual cycles during this characterization of stress sensitivity (i.e MSR and SS monkeys) were allowed to recover normal menstrual cyclicity before further experiments were resumed. One HSR and one SS monkey were euthanized for clinical reasons prior to this study. Therefore, this examination of neural gene expression contained five animals categorized as HSR, four animals categorized as MSR, and four animals categorized as SS.

2.3 Short-term stress

Following a rest period and resumption of normal menstrual cycles, each animal was subjected to the combined moderate stress paradigm described above *for 5 days* and then euthanized. Hence, each animal was euthanized on day 5 of the follicular phase.

2.4 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 pentobarbitol (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

The left ventricle of the heart was cannulated and the head was perfused with 1L of saline (made with DEPC-treated water [0.1% diethyl pyrocarbonate] to minimize RNase contamination) followed by 7 liters of 4% paraformaldehyde in 3.8% borate, pH 9.5 (Berod et al., 1981, Simmons et al., 1989). The brain was removed and dissected. Blocks of tissue containing the pontine midbrain were post-fixed for 3 hours in 4% paraformaldehyde, then washed in 0.02 M potassium phosphate-buffered saline (KPBS) containing first 10% (overnight), then 20% glycerol (48 h), plus 2% dimethyl sulfoxide (DMSO) to cryoprotect the tissue. The blocks were frozen in precooled methylbutane (-55°C) and stored at -80°C for up to 6 months. The midbrain blocks containing the dorsal raphe were cut on a sliding

microtome at 25 μm , and half of the sections were mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA), dehydrated under vacuum overnight and then stored at -80°C until processing for *in situ* hybridization (ISH) assays. The other half of the sections was stored at -20°C in cryoprotectant for subsequent immunohistochemical (IHC) assays.

2.5 Riboprobes

A monkey specific partial cDNA clone of TPH2 containing 251 bp of the 5' region, which has very little homology to TPH1, was previously constructed (Sanchez et al., 2005) and the TPH2 riboprobe incorporating digoxigenin was synthesized as previously described (Bethea et al., 2012b). New monkey specific partial cDNA clones of SERT and 5HT1A were constructed.

Based upon the rhesus monkey SERT sequence (NM_001032823), forward and reverse primers were ordered (Invitrogen, Carlsbad, CA) to amplify a 309bp sequence from 1230-1182 bp. The primer sequences were:

Forward: TGCAGAAGCGATAGCCCAACAT

Reverse: TTTCTTCACGTCCCTGCAG

A 309bp amplicon was generated from rhesus macaque dorsal raphe mRNA. The amplicon was inserted into the pGEM_T vector and sequenced. The cRNA antisense probe was generated by linearization with SAC I digestion and T7 transcription. The cDNA sense probe was generated by linearization with SAC II digestion and SP6 transcription.

Based upon the rhesus monkey 5HT1a sequence (NM_001198700), forward and reverse primers were ordered (Invitrogen, Carlsbad, CA) to amplify a 306bp sequence from 483-788 bp. The primer sequences were:

Forward: ACCAGCGGCAACACTACTGG

Reverse: GGACGAGGTGCAACACAGC

A 306bp amplicon was generated from rhesus macaque dorsal raphe mRNA. The amplicon was inserted into the pGEMT vector and sequenced. The cRNA antisense probe was generated by linearization with SAC II digestion and SP6 transcription. The cRNA sense probe was generated by linearization with SAC I digestion and T7 transcription.

The cRNA probes were labelled with digoxigenin according to established protocol. In-vitro transcription for cRNA was done in 500ng digested plasmid, 2mM Dig UTP (Roche Diagnostics, Indianapolis, IN), 5X Transcription buffer, NTPs (ACG) 2.5mM, DTT 10mM, RNA polymerase 20U (Promega, Madison WI) RNase inhibitor 40U (Ambion/Invitrogen) in 10ul reaction. After DNase treatment (5U/reaction) the quantity and quality of the digoxigenin-labelled probes was assessed with agarose gels and dot blot analysis. The cRNA probe was titrated for ISH assay as follows: TPH2 equalled 18ng/ul, SERT equalled 15ng/ul and 5HT1A equalled 20ng/ul.

2.6 In situ hybridization (ISH) assays

The non-isotopic *in situ* hybridization procedure, described in detail elsewhere (Berg-von der Emde et al., 1995, Bethea, Smith, 2012b, Lima, Centeno, 2009), utilized digoxigenin-labelled TPH2, SERT and 5HT1A cRNAs. It was applied to determine if mRNA abundance differs between HSR, MSR and SS monkeys after 5 days of moderate psychosocial and metabolic stress. The cRNA was prepared using cDNAs as template and SP6 RNA polymerase to drive the transcription reaction as described above (Berg-von der Emde, Dees, 1995). Following an overnight hybridization at 55-56 $^{\circ}\text{C}$, the slides were washed and

processed for digoxigenin detection of TPH2, SERT and 5HT1A as reported elsewhere (Bethea, Smith, 2012b). After developing the digoxigenin/antidigoxigenin-alkaline phosphatase conjugate reaction by an overnight incubation in a 4-nitrobluetetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate staining solution, the slides were extensively washed in potassium phosphate-sodium chloride buffer pH 7.4, dehydrated in graded ethanol, dried, and coverslipped for microscopic examination. Four levels of the dorsal raphe were analyzed at 250 μ m intervals. The sections were matched anatomically and rostral, medial and caudal sections from all animals were processed together.

2.7 cFos Immunohistochemistry

Floating sections were removed from cryoprotectant and washed in 0.02 M KPBS for 1 hour with buffer refreshments every 15 min. Next, the sections were incubated in 0.3% H₂O₂ in methanol for 30 min. and again washed in KPBS for 1 hour with buffer refreshments every 15 min. Nonspecific binding was blocked with incubations in 2% normal goat serum for 1 hour, in 5% avidin for 20 min, in 5% biotin for 20 min, and in 3% bovine serum albumin for 1 hour. Sections were then incubated in primary antibody against cFos for 48 hours (1/200; anti-cFos (4): sc52, Santa Cruz Biotech, Santa Cruz, CA). After primary incubation, the sections were washed as described above; incubated with secondary antibody anti-rabbit IgG for 1 hour (1/500; Vector anti-rabbit ABC kit, Vector Laboratories, Burlingame, CA); re-washed as above, incubated in Avidin-Biotin Complex for 1 hour (as recommended by Vector); re-washed as above; incubated with diaminobenzadine (DAB) for 10 min; re-washed as above and mounted on gelatin-subbed slides. After drying overnight under vacuum, the sections were dehydrated through a graded series of ethanol (3 min each); immersed in xylene for 15 min with 5 min changes and finally coated with Vector Mount and a coverslip.

2.8 Densitometric Analysis

Sections (4 levels/animal) were video-captured with the Marianas Stereology Workstation and Slidebook 5.0. A montage of the dorsal raphe at each level was created by Slidebook, which was subjected to further analysis with Image J. For each anatomical level, the largest representation of the dorsal raphe was chosen from amongst all of the animals. A square outline was placed over the chosen dorsal raphe and the exact dimensions were recorded. The same size square was then used for all of the animals at that anatomical level. First, the operator outlined (boxed) the dorsal raphe nucleus. Image J autosegmented the image into positive (stained) and negative (unstained) pixels. The program then provided the area of positive pixels, and with a filter applied, provided the number of cells in the defined area. The same procedure was applied with each gene at each anatomical level. The outlined area was also measured to ensure equal areas were analyzed across all animals. Therefore, for each section the following data were obtained: total area measured, positive pixel area, and cell number. Further calculation yielded the total and average cell number and positive pixel area.

2.9 Comparison Across Studies

It was of interest to compare earlier studies of animals that were euthanized in the absence of stress with this study of animals euthanized after 5 days of stress. The serotonin-related genes were previously examined in non-stressed HSR and SS animals with radioactive ISH and autoradiographic film analysis (Bethea et al., 2005b, Lima, Centeno, 2009). Therefore, the data from Lima et al, 2009 was further extracted for comparison to the data in this study. However, since this study was conducted with digoxigenin ISH, the absolute values could not be compared to the unstressed animals. Nonetheless, it was informative to examine the relative pattern of expression for each gene. The ratio of HSR/SS for each gene in the presence and absence of stress was obtained. Please note that the Lima et al 2009 study

examined only the HSR and SS groups so the HSR and SS groups from this study were used. These groups represent the extremes of the population, which are the most informative for comparison.

2.10 Statistical Analysis of ISH and IHC signals

All measurements were either totalled or averaged across the levels, generating one overall total or average for each animal. The data were compared with analysis of variance (ANOVA) followed by Newman-Keuls post hoc pairwise comparison. For further interpretation, the HSR and SS groups were compared with Student's t-test. The variance reflects the difference between animals. In addition, concordance between TPH2, SERT, 5HT1A and cFos was determined by calculation of Kendall's Coefficient of Concordance yielding Kendall's W. All statistical analyses were conducted using the Prism Statistic Program 5.0 (GraphPad, San Diego, CA). A confidence level of $p < 0.05$ was considered significant.

Results

3.1 Digoxigenin signal and appearance of dorsal raphe

The staining for TPH2-, SERT- and 5HT1A-digoxigenin is illustrated in Figure 1. Control sections processed without probe or with sense probe showed little non-specific staining. The staining is confined to the cytoplasm with each probe, consistent with labeling of RNA. The SERT probe produced relatively more staining than the TPH2 or 5HT1A. The anatomy of the sections from a rostral to a caudal level is illustrated in Figure 2 with SERT labeling. Four representative levels are shown. Panels A and B illustrate the 2 rostral levels and panels C and D illustrate the 2 mid-to-caudal levels that were subjected to image capture and analysis.

3.2 TPH2

The average TPH2-positive cell number and the average TPH2-positive pixel area across 4 sections were obtained for each animal. Then, the average of the animals in each group was obtained so the standard error of the mean reflects the animal-to-animal variation. Figure 3 illustrates the overall average TPH2-positive cell numbers (top) and the overall average TPH2-positive pixel area (bottom) in each group. There was a significant difference in average cell number across the 3 groups (top left; $F [df12]=3.880$; $p < 0.034$). The SS group was significantly lower than the HSR group (Newman-Keuls, $p < 0.05$). Similar results were obtained in comparison of the total cell number across the 3 groups (not shown; ANOVA $F[df12]=3.931$; $p < 0.05$). However, there was no difference in the average (bottom left) or total (not shown) TPH2-positive pixel area across the 3 groups. Nonetheless, with ANOVA differences between groups can be obscured when changes are gradual. Therefore, we compared the HSR group to the SS group directly. There was a significant difference between the HSR and SS groups in the average TPH2 positive cell number. The SS group had significantly fewer average TPH-2-positive cells than the HSR group (top right; $t [df7]=3.37$; $p < 0.011$). Similar results were obtained in comparison of the total cell number of 2 groups (not shown, $t [df7]= 3.389$; $p < 0.011$). In addition, the SS group had lower TPH2-positive pixel area compared to the HSR group on average, but this difference did not reach statistical significance (bottom right; $t [df7]=2.131$; $p < 0.07$). Similar results were obtained in comparison of the total positive pixel area (not shown; $t [df7]=2.131$; $p < 0.07$). Due to the small number of animals, this may be a type 2 statistical error.

3.3 SERT

The average SERT-positive cell number and the average SERT positive pixel area across 4 sections were obtained for each animal. Then, the average of the animals in each group was obtained so the standard error of the mean reflects the animal-to-animal variation. Figure 4 illustrates the overall average number of SERT-positive cells (top) and the overall average SERT-positive pixel area (bottom) in each group. There was no difference in the average SERT-positive cell number across the 3 groups (top left) or in the total SERT-positive cell number (not shown). However, the average SERT-positive pixel area was statistically different across the 3 groups (bottom left; $F [df12]=5.195$; $p < 0.028$), as was the total (not shown; ANOVA $F [df12]=5.196$; $p > 0.028$). The HSR group was significantly higher than the other groups in average and total (not shown) SERT positive pixel area (Newman-Keuls $p < 0.05$, both). Direct comparison of the HSR group to the SS group is illustrated on the right. There was no difference between HSR and SS groups in the average SERT-positive cell number (top right), or in the total SERT-positive cell number (not shown). However, the average SERT-positive pixel area was lower in the SS group than the HSR group, (bottom right; $t[df7]=2.131$; $p = 0.053$). Similar results were obtained in comparison of the total positive pixel area (not shown, $t [df7]=2.131$; $p = 0.053$).

3.4 5HT1A

The average 5HT1A-positive cell number and the average 5HT1A positive pixel area across 4 sections were obtained for each animal. Then, the average of the animals in each group was obtained so the standard error of the mean reflects the animal-to-animal variation. Figure 5 illustrates the overall average positive 5HT1A cell numbers (top) and the overall average 5HT1A-positive pixel area (bottom) in each group. There was no difference in the average 5HT1A-positive cell number across the 3 groups (top left), and there was no difference in the average 5HT1A-positive pixel area across the 3 groups (bottom left). There was also no difference in the total 5HT1A-positive cell number or -positive pixel area (data not shown). Direct comparison of the HSR group to the SS group is illustrated on the right. 5HT1A-positive cell numbers were lower in the SS group than the HSR group on average, but the difference did not reach statistical significance (top right; $t[df7]=2.137$; $p < 0.07$), which due to the small animal number may be a type 2 statistical error. Similar results were obtained in comparison of the total cell number (not shown, $t[df7]=2.136$; $p < 0.07$). However, the SS group had significantly lower 5HT1A-positive pixel area than the HSR group (bottom right; $t[df7]= 2.40$; $p < 0.05$). Similar results were obtained in comparison of the total positive pixel area (not shown; $t [df7]=2.340$; $p < 0.05$).

3.5 Comparison across experiments

Kendall's W (Coefficient of Concordance) for TPH2, SERT and 5HT1A equalled 0.54. This rejects the null hypothesis that there is no concordance ($W=0$), but it does not support complete concordance ($W=1.0$). Nonetheless, the coefficient indicates a fair degree of agreement.

We previously observed that the expression of TPH2, SERT and 5HT1A was significantly lower in (placebo treated) SS than HSR animals in the absence of stress. Original autoradiograms and data are shown in (Bethea, Streicher, 2005b, Lima, Centeno, 2009). The previous data was obtained with radiolabeled probe and autoradiographic film analysis. To compare the previous data with the current data, we used the average positive pixel area, and calculated the HSR/SS ratio for each gene, with and without stress. The results are illustrated in Figure 6. Since this ratio results from the division of one number into another number, there is no statistical test. However, it is evident that there is little or no difference in the ratio between animals examined in the absence of stress and animals examined after 5

days of moderate stress. Thus, the difference (delta) between the HSR and SS groups was similar in the presence and absence of stress.

3.6 cFos

cFos is an intermediate early gene that has been routinely used as a reflection of neuronal activity. We questioned whether gene expression reflected general neuronal activation in the dorsal raphe or in the neighboring periaqueductal gray area (PAG) of the different groups. Figure 7 shows representative cFos staining in the dorsal raphe. The cFos-positive cells are evident as dark black dots (arrows). The insert in the HSR panel shows cFos staining at a higher magnification. Of note, the large serotonin cells are evident from the background staining and marked with an asterisk. Figure 8 shows representative cFos staining in the PAG.

As shown in Figure 9, there was a decline across the 3 groups in cFos-positive cells in both the dorsal raphe (left) and PAG (right) as stress sensitivity increased, or as stress resilience decreased, but it did not reach statistical significance with ANOVA (raphe top left; $F[df11] = 3.510$, $p = 0.07$; PAG top right; $F[df11] = 3.804$, $p = 0.063$), which may reflect a type 2 statistical error. However, direct comparison of the HSR to the SS group showed that the SS group had significantly fewer cFos-positive cells in both the dorsal raphe (bottom left; $t[df6] = 2.576$, $p = 0.042$) and in the PAG (bottom right; $t[df6] = 2.725$, $p = 0.034$).

Discussion

4.1 Serotonin and stress in rodents

The serotonin system plays a pivotal role in mediating stress and the stress response (Lira et al., 2003, Maier and Watkins, 2005, Mo et al., 2008, Pare and Tejani-Butt, 1996, Savitz et al., 2009). Numerous studies have found that administration of a variety of selective serotonin reuptake inhibitor (SSRI) antidepressants prior to stress in rodents, ameliorates a wide variety of physiological and behavioral manifestations of stress (Abumaria et al., 2007, Adams et al., 2005, Alfonso et al., 2005, Berton et al., 1999, Watanabe et al., 1993). However, the precise reaction of the serotonin system to stress in rodents varies across studies with strain, type of stress and length of the stress period. An early study found an increase in serotonin in the dorsal raphe at 2 hrs after a formalin paw injection (Palkovits et al., 1976). Later, one hour of sound stress was shown to elevate TPH enzyme activity (Singh et al., 1990). Social defeat, considered an acute stress, increased c-fos expression in serotonin and non-serotonin neurons of the dorsal raphe (Martinez et al., 1998). Recently, an increase in TPH2 was found after social defeat in stress sensitive rats (Gardner et al., 2009). From these studies on stress, serotonin and TPH, one may surmise that in rodents, stress activates raphe neurons and increases serotonin. We examined this notion in monkeys.

With respect to the autoreceptor in rodents, uncontrollable social defeat, like inescapable shock, causes an increase in anxiety and a down-regulation of dorsal raphe 5HT1A receptors, and a 5HT1A-receptor antagonist prevented both (Short, 2003). In contrast, tissue specific conditional knock down of the 5HT1A autoreceptor in rodents increased vulnerability to stress (Richardson-Jones et al., 2010). Nonetheless, there are significant differences between rodents and primates.

4.2 Serotonin-related gene expression after short-term stress in macaques

After short-term stress, TPH2 expression was lower in stress-sensitive, SS monkeys compared to resilient, HSR monkeys. Although there are no direct comparative studies, the same result was obtained by this laboratory in HSR and SS animals in the absence of stress (Lima, Centeno, 2009).

The expression of SERT was also lower in the SS group compared to the HSR group. In depressed humans, perhaps like SS monkeys, SERT binding was lower than normal controls or presumably stress-resilient individuals (Malison et al., 1998, Parsey et al., 2006).

Finally, the expression of 5HT1A autoreceptor mRNA was lower in SS than HSR animals. The SS animals are by definition more sensitive to stress. So, these data suggest that reduced 5HT1A mRNA correlates with sensitivity to stress. The 5HT1A autoreceptor +/- knock down mouse may be likened to our SS monkeys who exhibit lower levels of 5HT1A mRNA in the raphe (Bethea, Streicher, 2005b, Lima, Centeno, 2009).

We found lower expression of Fev, the serotonin master gene in non-stressed SS animals compared to HSR animals (Lima, Centeno, 2009) and Fev expression drives TPH2, SERT and 5HT1A expression (Hendricks et al., 1999). The role of a master gene is further supported by the calculation showing concordance between expressions of the 3 genes in this study. Therefore, it follows that in SS animals, TPH2, SERT and 5HT1A gene expression is reduced relative to HSR animals and this relationship was apparently maintained with stress.

The functional consequences of gene expression need contemplation. In this study, the lower expression of the TPH2 gene points to lower expression of TPH enzyme and in turn, lower synthesis of serotonin in SS animals with stress. The relationship between extracellular serotonin and expression of SERT has received much attention in various species. While it is well accepted that SSRIs block SERT transport and elevate extracellular serotonin, this does not appear to replicate the physiological link between serotonin and SERT expression. In human depression, lower extracellular serotonin is accompanied by lower SERT binding. According to one model, when extracellular serotonin is high, SERT will continuously pump serotonin to the inside of cells and the translocation of serotonin prevents SERT phosphorylation and internalization (Ramamoorthy and Blakely, 1999, Ramamoorthy et al., 1998). There are trafficking-dependent and -independent regulations of SERT, which means the story is not as simple as 'lower SERT means higher serotonin' (Apparsundaram et al., 2001). So in this study, the lower expression of SERT is probably consistent with the lower production of TPH2 and serotonin.

4.3 Serotonin and 5HT1A decrease in contrast to dogma

We have observed significant differences in gene expression in two different cohorts of HSR and SS monkeys euthanized in the absence of stress, so we have reasonable confidence in asserting that SS monkeys start with significantly lower expression of TPH2, SERT and 5HT1A mRNAs than HSR monkeys (Bethea, Streicher, 2005b, Lima, Centeno, 2009). The lower level of 5HT1A expression in the SS group in the presence or absence of stress raises a conundrum in terms of our understanding of the regulation of 5HT1A. Given that 5HT1A autoreceptor expression is decreased by elevated serotonin in the extracellular space, why would animals with obviously lower serotonin production also have lower 5HT1A expression? We speculated that in this type of situation, the whole serotonin system in SS monkeys is abnormal due to developmental problems; and so the two groups of animals may be operating with a difference in serotonin homeostasis. In addition, studies of 5HT1A receptor binding in humans have indicated that 5HT1A receptors are lower in depression, a condition linked to lower overall serotonin levels and lower SERT binding (Drevets et al 1999, Moses-Kolko, 2008). This indicates that 5HT1A expression may be differentially regulated under different conditions. Moreover, this could mean that there is concordance in the expression of TPH2, SERT, and 5HT1A. Our analysis supported this possibility with $W=0.54$.

4.4 Comparison of serotonin-related gene expression in the presence and absence of stress

In our initial experiments, animals recovered from the stress of the characterization phase prior to euthanasia. After resumption of several normal menstrual cycles, we found significant differences in functional aspects of the serotonin system of macaques that were SS compared to HSR macaques. There was a reduction in global serotonin release in response to fenfluramine (Bethea, Pau, 2005a), and significantly lower levels of gene expression for TPH2, SERT, 5HT1A and Fev (the serotonin master gene) in SS animals compared to HSR animals in the absence of stress (Lima, Centeno, 2009).

In this study, macaques were characterized with our paradigm (Bethea, Centeno, 2008) as HSR, MSR or SS and allowed to recover menstrual cyclicity. Then, they were exposed to the same combined moderate stress *from day 1 to day 5* of the follicular phase of the menstrual cycle immediately prior to euthanasia. The outcome in the serotonin system indicated that serotonin-related gene expression decreased from HSR to MSR to SS groups in a pattern that was very similar to the non-stressed animals (Bethea, Streicher, 2005b, Lima, Centeno, 2009). Under optimal circumstances, gene expression would be examined in stressed and nonstressed groups at the same time and with the same type of assay. However, all raphe sections from the nonstressed animals were exhausted before the current experiment. Therefore, since non-stressed gene expression was obtained with other animals at another time and with a radiolabeled assay, we cannot make direct comparisons between the overall level of gene expression in the presence and absence of stress.

Nonetheless, it was possible to calculate a ratio, or the percent difference, in gene expression between the HSR and SS groups. This revealed that the difference between the HSR and SS groups was similar in the presence or absence of stress. Thus, in female macaques with different sensitivity to stress, moderate short-term stress did not appear to activate the serotonin system as observed in rodent studies. We hypothesize that the serotonin system differs with stress sensitivity, but it may not respond to short-term moderate stress. Although HSR and SS group differences may be maintained, it is conceivable that the mean of serotonin-related gene expression may have shifted. We cannot rule out the possibility that all subjects may have decreased or increased serotonin-gene expression to an equivalent degree in response to the short-term stressor.

In comparison, a limited measurement of the positive pixel area of the serotonergic innervation to the locus ceruleus was obtained in high and low resilient groups of non-stressed and stressed animals (Bethea et al., 2012a). We found that the boutons of the serotonergic innervation entering the locus ceruleus did not change with stress (in an area that was not compromised by dendrite swelling). That is, serotonin fibers were denser in the HSR than the SS group, and the values were the same in the presence and absence of stress implying that the serotonin system did not respond markedly to the short-term stress. However, the short-term stress significantly increased activity of the locus ceruleus, and the ratio of serotonin/NE strongly correlated with stress sensitivity. Altogether, the comparison of the ratios of serotonin gene expression agreed with the density of the serotonin innervation of the locus ceruleus ie., neither showed a difference in the presence or absence of short-term moderate stress. However, it is premature to declare that either observation provides conclusive data for the lack of a serotonergic response to short-term moderate stress in macaques and certainly longer-term stress may impact the serotonin system to a greater degree. Comparatively, stress had no effect on TPH activity in stress sensitive BALB/c mice either (Browne et al., 2011).

4.5 Longer-term or chronic stress

'Burn out' of the serotonin system with chronic stress has been suggested as one cause of depression. Palkovits and colleagues showed that following the increase in 5HT after acute stress, there was a failure of repeated stresses to elicit induction of serotonin (Palkovits, Brownstein, 1976). Therefore, another question remains regarding what occurs in the SS group with chronic stress leading to anovulation; and what would happen to the expression of the serotonin-related genes after chronic stress? Whatever happens, it happens within the first menstrual cycle because SS animals do not ovulate at all after initiation of stress. An experiment is underway in which HSR and SS animals will be exposed to our stress paradigm through 2 menstrual cycles (60 days) and concurrently treated with placebo or citalopram. The placebo group will approach these questions. It is also true that gene expression may not be the best method to determine serotonin functionality and other methods may be required. So far, we have examined gene expression in order to make comparisons between studies, which now span a period of ~12 years. Although we speculate that genetics and early life experiences underlie differences in sensitivity, we recognize the limitation of not having data on the developmental history of the animals. A study design in which we could control and experimentally manipulate the developmental experiences of the animals in order to analyze detailed development-gene-environment analysis would be optimal.

4.6 cFos expression

The detection of cFos expression paralleled serotonin-related gene expression in the dorsal raphe; and it was consistent with the serotonin system functioning at a lower level in the SS group compared to the HSR group. Although we have no sections for examination of cFos in the dorsal raphe of nonstressed monkeys, we hypothesize that similar results would be observed based upon equal serotonin fiber density in the locus ceruleus in the presence or absence of stress. A comparison to rodents presents some interesting parallels. Unlike monkeys, Adamec et al. (2012) reported an increase in cFos in the dorsal raphe after 2 kinds of stress, but whether the rats were more or less anxious had no effect (Adamec et al., 2012). In contrast, others found no increase in cFos in the dorsal raphe after stress even though fluoxetine prevented stress-induced increases in cFos in other areas of the brain (Lino-de-Oliveira et al., 2001). Another report found markedly lower levels of cFos in the rat hypothalamus after severe immersion or restraint stress (Briski and Gillen, 2001). However, exposure to a novel environment did not change the number of cFos-positive cells compared to nonstressed control rats (Briski and Gillen, 2001). It could be argued that 5 days of mild diet and novel environment in this monkey study are more similar to the novel environment stress in rats than immersion or restraint stress. These results also support our hypothesis that 5 days of moderate stress in monkeys does not increase activity or gene expression in the dorsal raphe. The PAG results are also interesting in that they suggest an underlying or endogenous difference in relay activity in the HSR and SS groups. Therefore, it will be important to determine if the higher expression of cFos in the HSR serotonin system is specific, or a general reflection of a more active brain overall.

4.7 Summary

In summary, the data from this study suggest that expression of serotonin-related genes and cFos in the dorsal raphe did not respond to 5 days of moderate stress, whether the monkeys were stress resilient or stress sensitive.

4.8 Conclusion

In conclusion, this study provides a unique view of serotonin gene expression in nonhuman primates with individual sensitivity to stress under short-term moderate psychosocial and

metabolic stress. It is clear that in the absence of stress, there is significantly lower expression of serotonin-related genes in SS animals compared to HSR animals (Bethea, Centeno, 2008). We now suggest that there is little to no adjustment in the serotonin system after 5 days of moderate stress. Therefore, we hypothesize that the serotonin system determines stress sensitivity, and we have other evidence that the noradrenergic system responds to stress. We further hypothesize that if this relationship is maintained, the relative function of these two systems may determine whether an individual exhibits FHA. These results point toward the possibility of pharmacological treatment for FHA.

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List of 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	dithiothreosine
DAB	diaminobenzidine
IgG	immunoglobulin G
PAG	periaqueductal gray
DEPC	RNase inhibitor for water treatment

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Highlights

- Serotonin-related gene expression is lower in stress-sensitive than resilient macaques.
- Delta of gene expression between groups is similar in presence or absence of stress.
- cFos detection is lower in stress-sensitive than resilient macaques.

Figure 1.

Representative photomicrographs of TPH2, SERT and 5HT1A digoxigenin ISH signal in the dorsal raphe at two different magnifications. The bar in the TPH2 pictures indicates the scale and it also applies to the photomicrographs of SERT and 5HT1A. The signal is restricted to the cytoplasm, which is the location of the majority of the mRNA. The SERT probe appears to detect more cells within any given area.

Figure 2.

Photomicrographs of SERT dig-ISH staining at the 4 anatomical levels used in this study as observed with the Marianas stereology workstation and Slidebook software. SERT staining was chosen for anatomical illustration because of the robust signal. Panels A-D illustrate the dorsal raphe anatomy in a rostral to caudal direction. Panels A and B illustrate the 2 rostral levels used and panels C and D illustrate the two caudal levels used. The representative sections are 250 μ m apart.

TPH2

Figure 3.

Histograms of the quantitative analysis of **TPH2** digoxigenin-ISH signal in all groups, and in the most stress resilient and most stress sensitive groups, HSR and SS, respectively. The **top** graphs illustrate the average positive cell number in each sensitivity group and the **bottom** graphs illustrate the average TPH2 positive pixel area in each sensitivity group. **Top left.** There was a significant difference between the 3 groups in the average TPH2 positive cell number (ANOVA $F [df12]=3.880$; $p < 0.034$). The SS group was significantly lower than the HSR group (Newman-Keuls, $p < 0.05$). **Top right.** There was also significant difference between the HSR and SS groups in the average TPH2 positive cell number (t

[df7]=3.37; $p < 0.011$). **Bottom left.** There was no difference between the 3 groups in the average TPH2 positive pixel area that could be discerned by ANOVA. **Bottom right.** There was a difference between the HSR and SS groups in the average TPH2 positive pixel area, but it did not quite reach statistical significance (t [df7]=2.131; $p < 0.07$). # strong trend * significantly different

Figure 4.

Histograms of the quantitative analysis of **SERT** digoxigenin-ISH signal in all groups *and* in the most stress resilient and most stress sensitive groups, HSR and SS, respectively. The **top** graphs illustrate the average positive cell number in each sensitivity group and the **bottom** graphs illustrate the average SERT positive pixel area in each sensitivity group. **Top left.** There was no difference between the 3 groups in the average SERT positive cell number that could be discerned by ANOVA. **Top right.** There was also no difference in average positive cell number between the HSR and SS groups. **Bottom left.** There was a significant difference between the 3 groups in the average SERT positive pixel area ($F [df12]=5.195$; $p < 0.028$). The HSR group was significantly higher than the other groups in average SERT positive pixel area (Newman-Keuls $p < 0.05$, both). **Bottom right.** There was also a significant difference between the HSR and SS groups in the average SERT positive pixel area ($t [df7]=2.321$; $p < 0.05$). * significantly different

Figure 5.

Histograms of the quantitative analysis of 5HT1A autoreceptor digoxigenin-ISH signal in all groups *and* in the most stress resilient and most stress sensitive groups, HSR and SS, respectively. The **top** graphs illustrate the average positive cell number in each sensitivity group and the **bottom** graph illustrates the average 5HT1A positive pixel area in each sensitivity group. **Top left.** There was no difference between the 3 groups in the average 5HT1A positive cell number that could be discerned by ANOVA. **Top right.** There was a difference between the HSR and SS groups in the average 5HT1A positive cell number that did not quite reach statistical significance ($t[df7]=2.137$; $p < 0.07$). **Bottom left.** ANOVA was unable to discern a difference between the 3 groups in positive pixel area. **Bottom right.** However, there was a significant difference between the HSR and SS groups in the average 5HT1A positive pixel area ($t [df7]= 2.40$; $p < 0.05$). # strong trend * significantly different

Figure 6.

Histograms depicting the ratio of the HSR/SS groups for each serotonin-related gene in the presence (black bars) and absence (white bars) of stress. In the absence of stress, positive pixel area of gene expression signal was obtained from autoradiograms, but in the presence of stress the positive pixel area of gene expression signal was obtained from images of digoxigenin-labeled cells. Statistics were not applied due to obtaining the ratio from the average positive pixel area of each gene, which did not yield variance. The difference between the HSR and SS groups was remarkably similar in the presence or absence of stress for each serotonin-related gene.

Figure 7.

Photomicrographs of cFos immunostaining in the dorsal raphe at level 3 as represented in panel C in Figure 2. The images were obtained with the Marianas stereology workstation and Slidebook software. Representative sections from an HSR, MSR and SS animal are shown. The small black dots represent cFos positive cells (black arrow). Serotonin neurons are visible from background staining as marked with an asterisk (*). The HSR section has the highest number of cFos positive cells. The insert contains a higher power image of the cFos staining obtained with a Leica brightfield microscope.

Figure 8.

Photomicrographs of cFos immunostaining in the PAG at level 1 or 2 as represented in panels A and B in Figure 2. The images were obtained with the Marianas stereology workstation and Slidebook software. Representative sections from an HSR, MSR and SS animal are shown. The small black dots represent cFos positive cells (black arrow). Aqueduct or central canal (AQ). The HSR section has more cFos positive cells than the MSR or SS sections.

Figure 9.

Histograms of the quantitative analysis of cFos immunostaining in the dorsal raphe and PAG. The **top** graphs illustrate the average number of cFos-positive cells in the HSR, MSR and SS groups after 5 days of stress (n=4/group). There was a near significant difference between the groups in the dorsal raphe and PAG (ANOVA F [df11]= 3.510; p = 0.07 and F [df11]= 3.804; p = 0.06, respectively). The **bottom** graphs compare the average number of cFos-positive cells in the HSR and SS groups only. The SS group had significantly fewer cFos-positive cells in the dorsal raphe and PAG than the HSR group (t [df6]=2.576; = < 0.04 and t [df6]=2.725; p = 0.03, respectively). # strong trend * significantly different