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Function and Innervation of the Locus Ceruleus in a Macaque Model of Functional Hypothalamic Amenorrhea

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Abstract

A body of knowledge implicates an increase in output from the locus ceruleus (LC) during stress. We questioned the innervation and function of the LC in our macaque model of Functional Hypothalamic Amenorrhea, also known as Stress-Induced Amenorrhea. Cohorts of macaques were initially characterized as highly stress resilient (HSR) or stress-sensitive (SS) based upon the presence or absence of ovulation during a protocol involving 2 menstrual cycles with psychosocial and metabolic stress. Afterwards, the animals were rested until normal menstrual cycles resumed and then euthanized on day 5 of a new menstrual cycle [a] in the absence of further stress; or [b] after 5 days of resumed psychosocial and metabolic stress. In this study, parameters of the LC were examined in HSR and SS animals in the presence and absence of stress (2 x 2 block design) using ICC and image analysis. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of catecholamines; and the TH level was used to assess by inference, NE output. The pixel area of TH-positive dendrites extending outside the medial border of the LC was significantly increased by stress to a similar degree in both HSR and SS animals ($p < 0.0001$). There is a significant CRF innervation of the LC. The positive pixel area of CRF boutons, lateral to the LC, was higher in SS than HSR animals in the absence of stress. Five days of moderate stress significantly increased the CRF-positive bouton pixel area in the HSR group ($p < 0.02$), but not in the SS group. There is also a significant serotonin innervation of the LC. A marked increase in medial serotonin dendrite swelling and beading was observed in the SS + stress group, which may be a consequence of excitotoxicity. The dendrite beading interfered with analysis of axonal boutons. However, at one anatomical level, the serotonin-positive bouton area was obtained between the LC and the superior cerebellar peduncle. Serotonin-positive bouton pixel area was significantly higher in HSR than SS animals ($p < 0.04$). There was no change in either group after 5 days of moderate stress. The ratio of serotonin/TH correlates with ovarian estrogen production

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with a sensitivity x stress interaction. Therefore, it appears that the serotonin system determines stress sensitivity and the NE system responds to stress. We hypothesize that elevated NE with low serotonin functionality ultimately leads to stress-induced infertility. In contrast, high serotonin functionality maintains ovulation in the presence of stress even with elevated NE.

Keywords

locus ceruleus; tyrosine hydroxylase; serotonin; CRF; stress; macaques; amenorrhea; ovulation; dendrites; excitotoxicity

Introduction

The syndrome known as Functional Hypothalamic Amenorrhea (FHA) is a cause of infertility in a significant subset of women presenting at infertility clinics. Approximately 15% of women desirous of children are infertile, and in 30% of these women (or 5% of all women) the diagnosis is FHA. This diagnosis is derived when there is no apparent anatomical, organic, or reproductive organ dysfunction/disease, but the women do not ovulate for 6 months (Gordon, 2010). An important body of clinical research has indicated that women with FHA feel significantly more stressed, but they do not necessarily have more stress in their lives than fertile women; that is, they appear to be more sensitive to stress (Berga et al., 1997; Berga and Gorton, 1989; Biller et al., 1990; Giles and Berga, 1993; Kondoh et al., 2001; Laughlin et al., 1998; Meczekalski et al., 2000).

Exposure to stressful stimuli can lead to a variety of secondary diseases such as anxiety, depression, cardiovascular disease, and immune suppression (McEwen, 2002). Now, reproductive dysfunction has been added to this growing list of stress-related disorders (Cameron, 2000; Xiao et al., 1999). Some of the neuroendocrine abnormalities associated with FHA are indicative of metabolic stress, and 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 strategies for coping with psychological stress and removal of metabolic stresses look very promising (Berga et al., 2003). Clearly, some individuals are very sensitive to stressors, while others are stress resilient. However, understanding the neurobiology underlying FHA requires a valid animal model that meets clinical criteria for FHA.

We have developed an experimental nonhuman primate model of FHA in which mild psychosocial stress combined with a mild diet, with or without a moderate exercise regimen, leads to a differential suppression of reproductive function that reverses upon stress removal (Bethea et al., 2008; Williams et al., 2007). 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). Stress-sensitive individuals also have higher basal heart rates throughout the 24 hr day compared to more stress-resilient animals (Cameron et al., 1998) and they have significantly lower peak estradiol levels in the follicular phase and significantly lower peak progesterone levels in the luteal phase of a normal, non-stressed, menstrual cycle compared to more stress-resilient animals (Bethea et al., 2008). Approximately one third of imported cynomolgus macaques in a given population fall into each category. However, attrition due to clinical reasons can change this distribution.

We have characterized aspects of serotonin function with this model. The release of serotonin in response to fenfluramine and pivotal serotonin gene expression was lower in SS animals compared to HSR animals (Bethea et al., 2008; Bethea et al., 2005). In addition, hypothalamic corticotropin releasing factor (CRF) gene and protein expression were higher in SS animals compared to HSR animals (Centeno et al., 2007); and this difference was further manifested by greater CRF-positive bouton area in the serotonergic dorsal raphe region in SS animals compared to HSR animals (Weissheimer, 2010).

The norepinephrine (NE) system also plays a manifest role in the stress response (Feder et al., 2009; Itoi and Sugimoto, 2010). The locus ceruleus (LC), located near the border of the pontine midbrain and medulla in primates, contains NE neurons that are part of the stress and anxiety circuit (Charney and Deutch, 1996) and LC neurons are stimulated by stress (Bonne et al., 2004). NE is synthesized by tyrosine hydroxylase (TH) and stress increases TH gene expression in rodents (Chang et al., 2000). Of note, increased NE neurotransmission from the A1 (ventrolateral medulla) and A2 (nucleus of the solitary tract) neurons enables the ovulatory surge of Luteinizing Hormone (LH) (Herbison, 1997). We questioned whether increased LC neurotransmission in response to stress may suppress ovulation and if so, how?

The majority of input to the LC comes from the autonomic nervous system (Aston-Jones et al., 1991c). In addition, the LC is innervated and stimulated by CRF (Curtis et al., 2012; Jedema and Grace, 2004; Valentino et al., 1993) and approximately 30% of CRF innervation to the LC comes from non-neuroendocrine neurons in the PVN (Reyes et al., 2005). A significant portion of the CRF innervation also derives from the paragigantocellularis (PGi) (Aston-Jones et al., 1991c). The LC also has serotonergic innervation, and the source of the serotonin neurons has been ascribed to the local pericoerulear area (Aston-Jones et al., 1991c; Miller et al., 2011). Others have suggested that as much as 50% of the serotonergic innervation of the LC originates in the dorsal raphe of rats (Kaehler et al., 1999; Kim et al., 2004). In general, serotonin appears to be inhibitory of LC activity (Aston-Jones et al., 1991a), but there is a complex interplay with other excitatory amino acids (Charlety et al., 1993) as well as different modes of NE discharge (Aston-Jones and Cohen, 2005).

We hypothesized that the LC input or activity may be different between stress resilient and stress sensitive monkeys. Moreover, the response of the LC to combined psychosocial and metabolic stress may vary depending on whether an individual is sensitive or resilient. To further this hypothesis, we examined the immunostaining of TH-positive dendrites surrounding the LC as a rudimentary reflection of noradrenergic activity; and we examined the CRF and serotonin innervations of the LC. These parameters were determined in monkeys characterized as sensitive or resilient and that were euthanized in the presence and absence of stress. Immunohistochemistry assays for TH, CRF and serotonin were applied to brainstem sections followed by quantitative image analysis of defined fields around the LC.

Materials and Methods

Animals and treatments

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

Thirty adult female cynomolgus monkeys (*Macaca fascicularis*) were utilized. The animals were 7–14 years of age. The animals were imported and immediately housed in single cages in the same room. No information is available regarding their social status prior to import. 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 four 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. There were no body weight differences throughout the characterization of stress sensitivity (Bethea et al., 2008; Herod et al., 2011a).

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 moderate psychosocial and metabolic stressor for up to 60 days, as described previously (Bethea et al., 2008). Sensitivity testing 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 (very early follicular phase) 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. Monkeys that menses 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). Dr. Cameron has established the need for combined life stresses for monkey FHA (Williams et al., 1997; Williams et al., 2007), which agrees with clinical literature (Berga and Loucks, 2005). The application of this protocol is referred to as the *characterization of stress sensitivity or resilience*.

Animals were categorized as HSR if they presented a normal ovulatory menstrual cycle [25–38 days in length, peak E₂ >200 pg/ml in follicular phase, peak P₄ >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 of stress cycle 1) were categorized as SS. All animals were returned to control conditions, rested and allowed to recover normal menstrual cyclicity *before the terminal protocol*.

Experimental Design

Two sets of animals (n=15/set) were characterized for determination of their sensitivity or resilience to stress as described above and then returned to control non-stress conditions and maintained until normal menstrual cycles resumed. Set 1 was euthanized in the absence of any further stress. This first set of animals was used in previously published studies on the dorsal raphe and hypothalamus and their characterization also included running on a treadmill [for review see Bethea et al., 2008]. Two animals were lost from Set 1 due to clinical reasons. Following a rest period and resumption of normal menstrual cycles, Set 2 animals were subjected to the combined moderate stress paradigm described above for 5 days and then euthanized. Set 2 is the same cohort of animals utilized in previous publications examining activity of the HPA axis (hypothalamic-pituitary-adrenal) in response to mild psychosocial and metabolic stress (Herod et al., 2011a) and to a corticotropin releasing factor receptor type 1 (CRF-R1) antagonist (Herod et al., 2011b). Two animals were lost from Set 2 for clinical reasons. All animals (set 1 and set 2, final n=13/set) were euthanized on day 5 of the follicular phase, defined as 5 days after the

initiation of menstruation. Thus, the Set 1 cohort was euthanized 5 days after menses in the absence of stress. Set 2 animals were re-stressed for 5 days after menses and then euthanized. The stress is considered short-term, moderate stress for nonhuman primates.

In this study of the LC, we utilized HSR and SS animals euthanized in the presence or absence of stress. The number of sections that can be run simultaneously in an assay necessitated eliminating the MSR groups. In addition, we decided to focus on the extremes of the population for revealing meaningful differences. Therefore, the final comparison was between 4 groups with a 2 X 2 block design. The groups were designated HSR-no stress (n=4); SS-no stress (n=4); HSR+stress (n=6), and SS+stress (n=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 pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

The left ventricle of the heart was cannulated and the head 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.

Pontine midbrain blocks containing the LC were cut on a sliding microtome at 25 µm; sections were placed in cryoprotectant (50% 0.05 M sodium phosphate buffer, 30% ethylene glycol, and 20% glycerol) and then stored at −20°C until processing with immunohistochemistry.

Immunohistochemistry

Midbrain sections were removed from cryoprotectant and −20 °C storage and washed in KPBS buffer 4 times for 15 min each, immersed in 1% hydrogen peroxide for 30 min, washed in KPBS buffer 4 times for 15 min each and then incubated with the following blocking solutions: Vector normal goat serum (NGS; Vector Laboratories, Burlingame, CA) for 60 min; 3% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 60 min; Vector avidin for 20 min; Vector biotin for 20 min. For CRF, sections were blocked with 0.1% human α-globulin (Sigma, St. Louis, MO) instead of BSA. Sections were then incubated for 48 h in primary antibody to tyrosine hydroxylase (TH, from Chemicon acquired by Millipore, Temecula, CA); corticotropin releasing factor (CRF; gift of Dr. Wylie Vale, Salk Institute, La Jolla, Calif., USA) or serotonin (Immunostar, Hudson, WI; formerly Incstar). They were extensively characterized across a range of titers with positive and negative controls; and were previously applied to primate brain (Bethea, 1994; Kohama et al., 1992; Sanchez et al., 2010). All of the primary antibodies were diluted in 2% NGS, 0.02 M KPBS, and 0.4% Triton. The TH antibody was diluted 1/1000; the CRF antibody was diluted 1/6,000 and the serotonin antibody was diluted 1/8000. Sections were then rinsed in KPBS buffer 4 times for 15 min each, incubated in Vector biotinylated goat anti-rabbit serum for 60 min, washed in KPBS buffer 4 times for 15 min each, incubated with Vector ABC reagent for 60 min, washed in KPBS buffer 4 times for 15 min each, incubated with 0.05% diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) containing 3% hydrogen

peroxide for 1–10 min, and finally washed in KPBS buffer 4 times for 15 min each. Sections were mounted on gelatin-coated slides and dried overnight under vacuum. The sections were further dehydrated through a graded series of ethanols and xylene. The sections were finally mounted under glass with DPX.

Each ICC assay contained one level from all animals, which equals 26 sections. This was a comfortable number of sections for technical processing. Therefore, the levels were matched anatomically using reference points and each assay contained one level from all animals.

Stereological Analysis of Immunostaining

Immediately after sectioning, a complete set of sections at 250 μ m intervals from each animal was stained with hematoxylin and eosin (H&E) for examination of the morphology of the entire LC. The sections were morphologically matched between animals using anatomical reference points. Several landmarks were pivotal and defined the LC from a rostral to a caudal level. The mesencephalic 5 tract (me5) was the best marker. It emerged at the most rostral level, then elongated into the LC and gradually merged with the LC becoming shorter and wider at our most caudal level (illustrated in Figure 1). The location of me5 relative to the superior cerebellar peduncle (branchium conjunctivum) was also useful. Although we cut in a slightly different plane, the sections are approximately located from Plate 90 (Interaural 00.30mm; Bregma –21.60mm) to Plate 94 (Interaural –01.50 mm; Bregma –23.40 mm) in the atlas called “The Rhesus Monkey Brain in Stereotaxic Coordinates (Paxinos et al., 2000). A Marianas Stereological workstation (Intelligent Imaging Innovations, Denver, CO) with Slidebook 4.2 (Olympus Imaging Systems, Center Valley, PA) was used for image capture. A montage of the entire area containing TH, CRF or serotonin immunostaining was built by the workstation. The Slidebook image was cropped and the illumination was adjusted with the software to the same level for all sections. The image was exported as a Tiff file and ImageJ (NIH freeware) was used to perform the analysis. The area containing staining of interest was defined, further cropped and kept constant across the levels and animals for each assay. Image J performed an automatic background subtraction using a ‘rolling ball’ with a preset radius of 50 pixels. After automatic speckle removal and contrast improvement to the same degree, the image was segmented into positive and negative pixels with the same saturation parameters. The positive pixel area was used for statistical analysis.

Statistical analysis

Four levels of the LC were analyzed for TH immunostaining and three levels of the LC were analyzed for CRF bouton immunostaining. For CRF and TH, the positive pixel area of the left and right sides of the brain were averaged for each anatomical level. Then, the average of the 3–4 levels was obtained, generating an average positive pixel area for each individual animal. One level of the LC in each animal was analyzed for serotonin bouton immunostaining due to an unexpected variation as presented in the results. For serotonin, the positive pixel area of the left and right sides of the section were summed and this constituted the individual animal data. The individual animal data was used for statistical comparison of the groups. Therefore, the variance within the groups reflects the difference between the monkeys. All data comparing the four groups were subjected to two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc pairwise comparison. Statistical analyses were conducted using the Prism Statistical software 5.0 (Graph-Pad Software, Inc., San Diego, CA, USA). A $p < 0.05$ was considered statistically significant.

Results

TH Immunostaining Increases with Stress

The immunostaining for TH was widespread and robust in the LC area. As noted by other investigators, the TH positive neurons of the LC have extensive dendrites, which extend from the nucleus and appear as a surrounding 'halo' (Shimizu et al., 1978; Swanson, 1976). This morphology is illustrated in Figure 1, panels A–D and at higher magnification in panel E (arrows point to dendrites). Panels A–D also illustrate the 4 anatomical levels of the LC that were analyzed in a rostral to caudal direction. Each level is 250 μ m apart. Since the sections are 25 μ m thick, this represents 850 μ m through the pontine midbrain. The area analyzed was located toward the upper medial portion of the nucleus as shown by boxes in panels A–D. The positive pixel area of the dendrites on the left and right sides of the brain was obtained and averaged for each anatomical level. Then, the average of the 4 levels was obtained, generating an average positive pixel area for each individual animal. Group means of the animals were statistically compared and the results are shown in Figure 2. There was a significant difference between the groups due to the presence and absence of stress ($p < 0.0001$) and a significant difference between the groups due to sensitivity status ($p < 0.01$), but no interaction. Thus, the SS animals had significantly higher TH-positive pixel area ($X \times 10^3$) than the HSR animals in the absence of stress (14.3 ± 0.8 vs. 12.4 ± 0.5); and stress caused a significant increase in TH-positive pixel area in both groups (both groups $p < 0.001$, Bonferroni's) with the SS animals reaching a higher absolute level (20.5 ± 0.8 vs 18.7 ± 0.4). Therefore, both HSR and SS animals increased TH with stress and by inference, increased NE transmission.

CRF Axonal Bouton Staining Increases with Stress only in Resilient Animals

The most consistent area for image analysis of CRF axonal fibers was located lateral to the LC as illustrated in Figure 3. Also observed were scattered CRF positive neurons. The presence of melanin in many of the neurons in the LC helped determine the anatomical level, but hindered analysis within the nucleus. In Figure 3, Panels A, B and C illustrate the anatomical levels that were analyzed for CRF immunostaining in a rostral to caudal direction. These correspond to levels 2, 3 and 4 of the TH analysis (panels B, C and D of Figure 1). Also shown in Figure 3, panel D is a higher power magnification of the area analyzed for CRF illustrating the CRF boutons that were counted (arrows) and a cell body that was erased (arrowhead) prior to counting. The positive pixel area of the CRF boutons on the left and right sides of the brain was obtained and averaged for each anatomical level. Then, the average of the 3 levels was obtained, generating an average positive pixel area for each individual animal. Group means of the animals were statistically compared and the results are shown in Figure 4. There was a significant interaction between the sensitivity status of the group and the response to stress ($p < 0.02$). There was a significant effect of stress ($p < 0.02$), but it depended on stress status. The SS group had a higher positive pixel area of CRF-immunostained boutons in the absence of stress compared to HSR (1490 ± 116 vs 1178 ± 126). However, in the presence of stress, the SS group exhibited no change in CRF-positive bouton area, whereas the HSR group showed an increase (1498 ± 167 vs 1781 ± 52).

Serotonin Axonal Bouton Area is Lower in SS animals and Does Not Increase with Stress

Serotonin immunoassay of the midbrain region containing the LC revealed a surprising variation between the groups, which limited analysis somewhat. We observed that the SS +stress animals had marked swelling and beading of serotonin dendrites, as shown in Figure 5. These swollen dendrites appear to derive from serotonin neurons that are medial to the LC and located along the wall of the ventricle. Panels A and B illustrate representative SS animals in the absence (A) and presence (B) of 5 days of moderate stress before euthanasia.

Light melanin deposits (little arrows) enabled designation of the LC. It is apparent from Panels B and C that the swollen serotonin dendrites extend into the body of the LC. Also apparent are beaded dendrites (arrowheads). Panel D illustrates a serotonin neuron near the LC with a swollen, beaded dendrite at higher power.

The area analyzed for serotonin bouton area is shown in Figure 6, top panel and is located at level 3 lateral to the LC between the lower LC and the superior cerebellar peduncle. This area was sufficiently large and consistent enough for image analysis. In Figure 6, bottom panel, the serotonin bouton staining is illustrated at higher power. Only the darkly staining boutons were segmented for measurements. The left and right sides of the lateral serotonin axons at level 3 were summed for each animal. The group means of the individual animals was used for statistical comparison. As shown in Figure 7, the pixel area of serotonin-positive boutons was significantly higher in HSR than SS animals ($p < 0.045$). However, there was no effect of stress on the pixel area of serotonin-positive boutons in either group, nor was there any interaction between sensitivity and the stress response. Analysis of an area outside the top of the body of the LC at level 2 and level 3 showed no difference between the groups. However, contamination with serotonin dendrite varicosities was a concern.

The Ratio of 5HT/TH correlates with Peak Serum Estradiol

Linear regression analysis was applied to determine if any of the neurotransmitters under study correlated with ovarian function. The late follicular surge in estradiol is necessary to trigger the LH surge, which induces ovulation. Therefore, ovarian function was defined as peak serum estradiol during a control, non-stressed menstrual cycle. Peak estradiol concentrations were previously published and are shown in Figures 2 and 4 of Bethea et al., 2008. Neither TH, serotonin (5HT) nor CRF predicted ovarian function, with or without stress, in the HSR and SS animals. The correlation between each neurotransmitter and the peak of estradiol during 'control 1' menstrual cycles was not significant. Therefore, different ratios of the transmitters were examined including 5HT/TH, CRF/TH, 5HT/CRF and the inverses. In addition, various combinations of the three measurements were examined including TH/(5HT/CRF), 5HT/(TH/CRF) and CRF/(TH/5HT), etc. Only the ratio of 5HT/TH reflected ovarian estradiol production across the 4 groups and correlated with peak serum estradiol from Bethea et al., 2008 as shown in Figure 8. The correlation coefficient $r^2 = 0.98$ and $p = 0.0064$.

Discussion

General

The noradrenergic neurons of the LC are thought to relay sensory and autonomic information to the thalamus, which in turn projects to cortical areas involved in a wide variety of emotional responses (Aston-Jones et al., 1991c; Bonne et al., 2004). The activation of the LC in response to stress or fear promotes heightened vigilance (Aston-Jones et al., 1991b) and two known outcomes of LC activation are aggression and anxiety (Charney, 2004; Haller et al., 1998; Marino et al., 2005).

We found that TH levels in the LC are chronically higher in SS than HSR animals without stress and TH increases significantly with 5 days of moderate psychosocial and metabolic stress. The serotonergic and CRF innervations of the LC did not appear to regulate TH, nor did TH, CRF or serotonin correlate with ovarian function. However, the ratio of 5HT/TH reflected peak serum estradiol of a normal menstrual cycle in the four groups as previously described (Bethea et al., 2008). Together, the data suggest that the interplay of the serotonin and LC systems may play a role in the mediation of stress-induced infertility.

TH Regulation, Anxiety and Agitation

The majority of TH immunostaining is found in dendrites and changes in TH immunostaining likely reflect changes in TH concentrations in the dendrites, which in turn could lead to greater production and transmission of NE. In the area surrounding the LC, the pixel area of TH-immunostaining in SS animals was higher compared to HSR animals without stress. With stress, the SS animals reached an even higher absolute TH-immunostaining level than the HSR animals, but it appeared that both groups sensed and responded to the stress with an increase in TH and presumably, LC activity. This data concurs with a body of knowledge regarding activation of the LC and an increase in TH gene expression in response to stress (Bonne et al., 2004). Of note, the LC also receives phenylethanolamine N-methyltransferase (PMNT)-containing terminals that would also stain for TH. However, this contribution would be quite small and it is unlikely to change the results (Chen et al., 2012; Milner et al., 1989).

Thus, the observed differences in TH in the dendrites of the LC reflected innate sensitivity and/or the presence or absence of stress. This suggested that the SS animals may be endogenously more anxious and indeed, this appears to be the case. Previously, we performed a series of temperament tests with female monkeys undergoing reproductive studies, to evaluate each monkey for several types of anxious behaviors. Behaviors such as excessive worry or fear (Kalin and Shelton, 2003; Schulkin, 2006), inhibited approach to novelty/hypoactivity (Biederman et al., 1990; Fox, 2004; Kalin and Shelton, 2003) and increased agitation/hyperactivity in response to novelty (Kelly et al., 1997; Shroff et al., 2006; Strekalova et al., 2005) have all been observed in both clinical tests and animal models of anxiety. These behavioral temperamental traits were examined in each monkey using tests developed for other studies and previously published (Bethea et al., 2004; Coleman et al., 2003; Williamson et al., 2003). In two of the tests, monkeys were placed in a novel setting: (1) the initial period of the Free Play test and (2) in the initial ALONE period of the Human Intruder test. In both of these periods, SS monkeys showed significantly more behavioral agitation, as measured by undirected activity, compared to HSR monkeys (Herod et al., 2006). The pathophysiologic mechanisms underlying psychomotor agitation include dysregulation of noradrenergic, serotonergic and GABAergic systems (Lindenmayer, 2000), all of which show differences between SS and HSR animals. Hence, our demonstration of greater TH production in SS animals under non-stressed conditions is consistent with the tendency of SS animals to show more behavioral agitation in novel situations.

CRF Innervation of the LC

The pixel area of CRF-positive boutons near the LC under non-stressed conditions was higher in the SS than HSR animals. This is consistent with our previous publication showing that the pixel area of CRF-positive boutons was higher in the dorsal raphe of SS compared to HSR animals (Weissheimer, 2010). However, with stress, the HSR group responded with a significant increase in CRF whereas the SS group did not respond. The study of CRF-positive boutons in the raphe of the stressed cohort is underway at this time so we do not currently have comparative data from the raphe. However, in several animal models and in some human studies, resilience appears connected to rapid activation of the stress response. Furthermore, resilience correlates with the capacity to limit stress-induced increases in CRH (Feder et al., 2009). Our data support the notion that stress-resilient animals have a rapid increase in CRF. Further study is needed to determine if subsequent termination occurs in the HSR animals. It is also possible that CRF has chronic and acute roles. Different concentrations of CRF appear to recruit different types of CRF receptors. For example, CRF under chronic conditions may activate CRF type 1 receptors (CRF-R1), which are thought to be anxiogenic. Perhaps with an acute elevation, CRF would activate CRF-R2 receptors as well, which are thought to be anxiolytic (Lukkes et al., 2008; Reul and Holsboer, 2002).

According to this line of reasoning, the HSR animals might be calmer under stress whereas the SS animals might be anxious and agitated. This speculation also agrees with our earlier temperament data (Herod et al., 2006). However, it is imperative to see the results of a longer period of stress for full interpretation of this response.

Another related observation found that male, but not female rats, exhibit a stress-induced CRF-R1 association with β -arrestin 2 that leads to receptor internalization in the LC. Because internalization is considered protective, its lack was hypothesized to contribute to the increased vulnerability of females to develop stress-induced pathology (Bangasser et al., 2010; Bangasser et al., 2012).

Serotonin Innervation of the LC

The serotonin bouton immunostaining in the lateral LC area was significantly lower in the SS than HSR animals in the absence of stress. This data is consistent with our report that SS animals have lower TPH, SERT and Fev (master gene) expression in the dorsal raphe under non-stressed conditions (Lima et al., 2009). In addition, serotonin is thought to be inhibitory of LC activity (Segal, 1979), and so the serotonergic innervation may play a role in TH regulation under non-stressed conditions. That is, the HSR animals have higher serotonin that may contribute to their lower TH, and the SS animals have lower serotonin that may contribute to their higher TH.

However, the serotonergic response to stress was unexpected, both in the marked increase of serotonin dendrite swelling and beading in the vicinity of the LC of SS animals; and in the lack of an afferent serotonergic response to stress in the lateral area of LC in either group. We have examined dorsal raphe serotonin-related gene expression in the presence of stress (manuscript in preparation), and it appeared that the SS+stress group was consistently lower than the HSR group, and the overall expression was little changed compared to groups without stress. This data is consistent with the serotonin innervation of the LC.

The presence of marked serotonin dendrite swelling and beading in the SS+stress group was a surprise. Dendrite swelling and beading are signs of excitotoxicity (Emery and Lucas, 1995; Hasbani et al., 1998). Glutamate excitotoxicity results in injury and cell death, and one important early sign of glutamate excitotoxicity is dendrite injury. Excitotoxic dendritic injury is characterized by the formation of focal swellings (varicosities) and dendritic varicosities have been demonstrated *in vivo* under pathological conditions (Hsu and Buzsaki, 1993; Olney, 1971). It has been suggested that varicosities may form when organelles and cytoplasm accumulate as the result of transport disruption and that protease activation by calcium influx leads to digestion of membrane stabilizing proteins (Emery and Lucas, 1995; Hasbani et al., 1998). To our knowledge, this is the first demonstration of serotonin dendrite injury with combined psychosocial and metabolic stress in a nonhuman primate. Of interest, Valentino and colleagues showed that a significant percent of CRF terminal boutons contain glutamate in the LC (Valentino et al., 2001). If similar synapses occur on serotonin neurons, then an increase in stress could increase glutamate to the point of excitotoxicity for serotonin neurons. We have shown increased DNA fragmentation, a harbinger of cell death, in serotonin neurons after ovariectomy of female macaques (Lima and Bethea, 2009). This study further suggests that chronic stress may lead to serotonin cell death in sensitive animals. However, dendrite varicosity formation can reverse after a very short stress *in vitro* (Park et al., 1996).

There remain some questions regarding the origin of the serotonin fibers innervating the LC. If the area examined has few axons from the dorsal raphe (Aston-Jones et al., 1991c), then the serotonergic innervation of the LC may be independent of the raphe during stress. However, we find considerable correlation between raphe gene expression (manuscript in

preparation) and LC serotonergic innervation in this study. If up to 50% of the serotonin axons in the LC area originate in the dorsal raphe (Kaehler et al., 1999; Kim et al., 2004), it may contribute to our results. Nonetheless, in addition to the raphe, serotonin neurons from other areas likely contribute to stress sensitivity and innervate the LC. Many factors other than gene expression impact neurotransmission and significant plasticity may ensue with longer periods of moderate stress.

Circuit Considerations

Serotonin and CRF regulate the activity of the LC under several different paradigms in rodents (Aston-Jones et al., 1991a; Curtis et al., 2012; Reyes et al., 2005). However, in this study there was no correlation between TH and CRF, or between TH and serotonin, suggesting that their innervation of the LC system was not profoundly determining TH with only 5 days of moderate stress. If TH immunostaining can be used as a surrogate marker for LC activity, then these data imply that other inputs to the LC are driving its activity to a greater degree. The major inputs to the LC are autonomic and sensory (Aston-Jones et al., 1991c; Reyes et al., 2005), suggesting that in this primate model of stress-induced infertility, the autonomic nervous system plays a sizeable role in ramping up the LC. Serotonin is thought to inhibit the LC under stimulatory conditions (Aston-Jones et al., 1991a; Charley et al., 1993). However, the serotonin innervation did not change with this type of stress and TH increased to a similar degree in both groups with stress.

Since no one system correlated to ovarian function we attempted to relate the systems to each other. To this end, we examined multiple ratios of each system to the others. Under non-stressed conditions, the ratio of serotonin to TH (5HT/TH) was lower in SS animals than HSR animals. With stress, 5HT/TH declined even further. This pattern resembles the pattern of ovarian steroid secretion in this model of FHA. That is, HSR animals start with significantly higher secretion of estradiol and progesterone during a normal non-stressed cycle. Upon stress, the secretion of estradiol and progesterone declines significantly in both groups. However, the absolute serum concentrations in the HSR animals are sufficient to elicit an ovulatory surge of LH, whereas estradiol and progesterone are completely suppressed in the SS group and there is no LH surge (Bethea et al., 2008). Moreover, there was a significant correlation between the 5HT/TH ratio and peak estradiol levels in control cycles. None of the other ratios resembled ovarian function across the 4 groups. Assembling ratios of the 3 endpoints in various orders was not enlightening.

An enticing hypothesis derived from this study is that serotonin underlies the sensitivity or resilience of the animal and NE reflects the stress response. In this respect, stress-induced NE on top of higher serotonin yields the resilient reproductive response and the HSR animals continue to ovulate. In contrast, stress-induced NE on top of lower serotonin in the SS animals suppresses ovulation and yields infertility. How this information is transduced to the hypothalamic-pituitary-gonadal axis is of significant clinical importance.

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Highlights

Cynomolgus macaques can be stress sensitive (SS) or highly stress resilient (HSR).

We examined TH, CRF, and serotonin (5HT) near the locus ceruleus.

Basal TH, CRF and 5HT were different between SS and HSR animals.

Stress-induced TH, CRF and 5HT were different between SS and HSR animals.

The 5HT/TH ratio correlated with peak serum estradiol.

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Figure 1.

Photographs of the TH dendritic immunostaining of the LC and the areas selected for image analysis and quantification (boxes). **Panels A–D** show montages of representative TH-stained sections at our designated anatomical levels 1–4 in a rostral to caudal direction as constructed by the Marianas workstation. **Panel E** shows a higher magnification of the TH positive cells in the body of the LC and the dendrite extensions that were measured as positive pixel area. Arrows indicate typical TH-positive dendrites. The images are from different animals that had the best representation of each particular level. Abbreviations: scp—superior cerebellar peduncle; me5 – mesencephalic 5 tract.

Figure 2.

Histogram illustrating the average TH positive pixel area extending medially from the LC. There was a significant difference between the groups due to sensitivity ($p < 0.0108$) and stress ($p < 0.0001$) with 2-way ANOVA. Stress caused a significant increase in TH immunostaining in both groups ($p < 0.001$, Bonferroni's posthoc test). HSR-no stress ($n = 4$); SS-no stress ($n = 4$); HSR+stress ($n = 6$), and SS+stress ($n = 4$).

Figure 3.

Photographs of the CRF immunostaining adjacent to the LC. The lateral areas selected for image analysis and quantification are shown with boxes. **Panels A–C** show montages of representative CRF-stained sections at our designated anatomical levels 2, 3 and 4 in a rostral to caudal direction as constructed by the Marianas workstation. **Panel D** contains a higher magnification of the area analyzed. It shows CRF positive boutons that were measured as positive pixel area, and a CRF positive cell body that was erased prior to measurement. The boutons were darkly stained beads averaging 3–4 pixels in size. The dark gray matter in the body of the LC is melanin. The small black cells are CRF positive. The images are from different animals that had the best representation of each particular level. Abbreviations: scp – superior cerebellar peduncle; me5 – mesencephalic 5 tract.

Figure 4. Histogram illustrating the average CRF bouton positive pixel area in an area lateral to the LC. There was a significant difference between the groups due to stress ($p < 0.02$) and there was a significant interaction between innate sensitivity and applied stress ($p < 0.02$) with 2-way ANOVA. Stress caused a significant increase in CRF only in the HSR group ($p < 0.01$, Bonferroni's posthoc test). HSR-no stress ($n = 4$); SS-no stress ($n = 4$); HSR+stress ($n = 6$), and SS+stress ($n = 4$).

Figure 5. Several photomicrographs of serotonin immunostaining that illustrate serotonin dendrite swelling and beading in an area medial to the LC. **Panels A and B** are low magnification montages of the LC and surrounding area obtained with Slidebook 5.0 and the Marianas workstation. **Panel A** contains a representative section from an SS–no stress animal. The LC (circle) was defined based upon the light melanin staining in the cell bodies of the nucleus and anatomical markers. The area medial to the LC is clear of large varicosities and largely contains serotonergic axonal boutons. **Panel B** contains a representative section from an SS +stress animal. The LC (circle) was defined based upon the light melanin staining in the cell bodies of the nucleus and anatomical markers. The area medial to the LC exhibits swollen dendrites with varying degrees of beading. The arrowhead points to a prominent serotonergic beaded dendrite. **Panel C** contains a color picture of the same section shown in Panel B at a slightly higher magnification. The LC is circled and arrows point to cells within the LC that contain melanin. This was used to identify the body of the LC (dashed circle). Arrowheads in the lower right corner point to prominent serotonergic dendrites with marked degrees of beading. **Panel D** illustrates a serotonin neuron in the area medial to the LC of an SS+stress animal at higher power with one prominent dendrite with large varicosities. This illustration was constructed of photomicrographs at several z-planes through the neuron. Abbreviations: scp – superior cerebellar peduncle; me5 – mesencephalic 5 tract.

Figure 6.

Top. Photograph of the area lateral to the LC that was quantified for serotonin axonal boutons from an HSR–no stress animal. The area is located at anatomically defined level 3. The LC of this animal contains many cells with heavy melanin deposits (**m**-arrow). There was significant variation of melanin deposits between animals. The square illustrates the area that was subjected to quantitative image analysis, which yielded the positive pixel area of the serotonergic boutons. The **bottom panel** contains a photomicrograph of the serotonin boutons at a higher magnification. The boutons were darkly stained beads averaging 3–4 pixels in size. The arrowhead points to a bouton. The images were from an HSR animal. Abbreviations: scp superior cerebellar peduncle; me5 mesencephalic 5 tract.

Figure 7.

Histogram illustrating the average serotonin bouton positive pixel area across the 4 experimental groups. There was a significant difference between the groups due to stress sensitivity ($p < 0.04$, 2 way ANOVA). There was no effect of stress and no interaction. HSR-no stress ($n = 4$); SS-no stress ($n = 4$); HSR+stress ($n = 6$), and SS+stress ($n = 4$).

Figure 8.

Top. Histogram illustrating the ratios of the average serotonin positive pixel area to the average TH positive pixel area (5HT/TH). The ratio of 5HT/TH is lower in the SS animals without stress and diminishes with in both groups with stress. This pattern resembled peak ovarian serum estradiol as previously measured under control and stress conditions in the Set 1 animal characterization (Bethea et al., 2008). **Bottom.** The linear regression analysis indicates that there is a significant correlation between the 5HT/TH ratio and peak serum estradiol concentrations in the presence and absence of stress.