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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 (2×2 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 with a sensitivity × 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.

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 and Girton, 1989; Berga et al., 1997; Biller et al., 1990; Giles and Berga, 1993; Kondoh et al., 2001; Laughlin et al., 1998; Meczekalski et al., 2000).

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,

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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 h 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 and 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., 2005, 2008). 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 et al., 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 Eight 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 ($32\times24\times27$ 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 mensed 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, 2001). Dr. Cameron has established the need for combined life stresses for monkey FHA (Williams et al., 1997, 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 $E_2>200$ 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