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Hypothalamic KISS1 Expression, GnRH, and Neurotransmitter Innervation Vary with Stress and Sensitivity in Macaques

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Abstract

This study examined the effect of short-term psychosocial and metabolic stress in a monkey model of Stress-Induced Amenorrhea on the HPG axis. KISS1 expression was determined with ISH in the infundibular arcuate nucleus (INF ARC). Downstream of KISS1, GnRH axons in lateral areas rostral to the infundibular recess, serum LH and serum oestrogen (E) were measured with IHC and RIA. Upstream of KISS1, norepinephrine (NE) axons in the rostral ARC and serotonin axons in the anterior hypothalamus and periaqueductal gray (PAG) were measured with IHC. Female cynomolgus macaques (*Macaca fascicularis*) characterized as highly stress resilient (HSR) or stress sensitive (SS) were examined. After characterization of stress sensitivity, monkeys were either not stressed, or mildly stressed for 5 days prior to euthanasia in the early follicular phase. Stress consisted of 5 days of 20% food reduction in a novel room with unfamiliar conspecifics. There was a significant increase in KISS1 expression in HSR and SS animals in the presence versus absence of stress ($p=0.005$). GnRH axon density increased with stress in HSR and SS animals ($p=0.015$), while LH showed a gradual, but non-significant increase with stress. E trended higher in the HSR animals and there was no effect of stress ($p=0.83$). NE axon density (marked with dopamine β -hydroxylase, DBH) increased with stress in both HSR and SS groups ($p=0.002$), whereas serotonin axon density was higher in HSR compared to SS animals and there was no effect of stress ($p=0.03$). The ratio of DBH/E correlated with KISS1 ($p=0.052$), and GnRH correlated with serum LH ($p=0.039$). In conclusion, E inhibited KISS1 in the absence of stress, but stress increased NE, which may override E inhibition of KISS1 expression. We speculate that neural pathways transduce stress to KISS1 neurones, which changes their sensitivity to E.

Keywords

Stress; resilience; KISS1; GnRH; norepinephrine; serotonin; LH; oestrogen; amenorrhea; macaques

Introduction

Kisspeptin is a neurohormone that is coded by the KISS1 gene and it is produced in widespread populations of neurones (1–4). The neuronal populations in the arcuate nucleus (ARC) and in the anterior region of the hypothalamus are regulated by oestradiol (E) in all species examined to date. Kisspeptin acts through the GPR54 receptor, which is expressed by GnRH neurones. We now understand that kisspeptin neurones stimulate GnRH neurones, that kisspeptin neurones have oestrogen and progesterone receptors (ER, PR), and that E can both reduce KISS1 gene expression in a negative feedback manner and increase KISS1 expression in a positive feedback manner prior to the ovulatory surge of LH [reviewed in (5–7)]. These actions of kisspeptin give it an important, and perhaps penultimate, position in the hierarchy of neuronal systems that regulate reproductive function.

Functional Hypothalamic Amenorrhea (FHA), also called Stress-Induced Amenorrhea, is a form of infertility in which the hypothalamic-pituitary-gonadal (HPG) axis is suppressed and menstrual cycles are lacking or irregular without ovulation (8). We have developed an experimental nonhuman primate model of FHA in which mild psychosocial stress combined with a mild diet, plus or minus a moderate exercise regimen, leads to suppression of reproductive function to different degrees that reverses upon stress removal (9–11). Female cynomolgus monkeys are either [1] highly stress-resilient (HSR) and maintain normal menstrual cyclicity and ovulation 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, similar to women with FHA (9, 10).

Dysfunctions of the CRF, noradrenergic (NE) and serotonin systems are common in mood or anxiety disorders, and play critical roles in the stress response (12, 13). In addition, the CRF, NE and serotonin systems provide input into the HPG axis and affect reproductive potential (14). We have previously demonstrated that there are pivotal differences between HSR and SS animals in functional aspects of the CRF, NE and serotonin systems in control menstrual cycles and after moderate psychosocial and metabolic stress (11, 15–18).

In a manner that is not totally defined, these systems impinge upon the neurones regulating reproduction, such as GnRH and kisspeptin neurones or their immediate upstream afferents (19, 20). We previously showed that GnRH expression in cell bodies of HSR and SS animals inversely correlated with serum E (11). We questioned whether KISS1 gene expression would show differences between HSR and SS animals in the presence and absence of stress. In addition, we examined reproductive hormones downstream of KISS1 including GnRH, serum LH and serum E. We also examined upstream neurotransmitters norepinephrine (NE), marked by dopamine β -hydroxylase (DBH), and serotonin. Correlations between KISS1 and upstream or downstream parameters were sought.

Methods and Materials

Animals and treatments

This experiment was approved by the Institutional Animal Care and Use Committee 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.

Adult female cynomolgus monkeys (*Macaca fascicularis*) were imported for study. Untreated animals (cohort 1) and placebo-treated animals (cohort 2) were euthanized on day 5 of the follicular phase in the absence of stress. Animals in cohort 3 were euthanized after 5 days of moderate psychosocial and metabolic stress on day 5 of the follicular phase. The animals were 7–13 years of age. Upon receipt, the animals were immediately housed in single cages in the same room. Cynomolgus macaques form social hierarchies in grouped housing and subordinates receive more aggression. With single cages, the rank is not a variable. Housing, diet, food intake, and daily vaginal swabs for detecting menstruation have been described previously (21, 22). The monkeys were similar in weight, and there were no body weight changes throughout the characterization of stress sensitivity (23).

These are the same animals utilized in previous publications that [1] established the characterization protocol and examined the underlying neurobiology in the absence of stress (11); [2] examined reproductive and neurobiological responses to placebo or citalopram under non-stressed conditions (15, 24–26), and [3] characterized adrenal and neurobiological responses to stress (16–18, 23, 27).

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. Characterization of sensitivity was performed after each monkey had been living in its home cage surrounded by familiar monkeys for several months and exhibiting regular menstrual cycles. The stressor consisted of moving the monkeys to a novel room surrounded by unfamiliar conspecifics and reducing the available chow by 20%, as described previously (11). 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.

Animals were categorized as HSR if they presented a normal ovulatory menstrual cycle [25–38 days in length, peak E >200 pg/ml in follicular phase, peak P >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 or mense within 60 days) were categorized as SS. All animals were allowed to recover normal menstrual cyclicity before further experimentation. Two monkeys were euthanized due to clinical issues from cohorts 1 and 3. For neurobiological studies, 4 HSR and 4 SS animals from cohort 1 were euthanized in the absence of stress, but sections from 1 HSR animal were damaged; 4 HSR and 4 SS

placebo-treated control animals from cohort 2 were euthanized in the absence of stress; and 6 HSR and 5 SS animals from cohort 3 were euthanized in the presence of stress. The number of animals available for each assessment may vary from the number euthanized due to variation in section integrity.

Only HSR and SS animals were utilized for a 2×2 block design due to the technical difficulties encountered with processing many sections in one assay. These groups represented the extremes of sensitivity, thereby providing clear differences in the presence and absence of stress.

Animals with No stress versus Short-term stress

Following a rest period and resumption of normal menstrual cycles, the first non-stressed cohort 1 was euthanized on day 5 of the follicular phase in the fall of 2001. The placebo-control animals of cohort 2 were euthanized in the absence of stress in the fall of 2003 on day 5 of the follicular phase. The stressed animals of cohort 3 were re-subjected to the combined moderate stress paradigm described above *from day 1 through day 5* and then euthanized on day 5 of the follicular phase in the fall of 2008. Due to funding cycles, it was not possible to process the different cohorts of monkeys at the same time and a longitudinal approach was required.

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 details of the brain perfusion, dissection, postfixation, cryoprotection, sectioning, mounting and storage were previously described (17). For this study, the hypothalamic blocks were sectioned at 25 μm intervals yielding 10 representative sets containing 25 sections at 250 μm intervals. Half of the sets were immediately mounted and saved at -80°C . The other half were stored floating in cryoprotectant at -20°C .

RNA degradation

The time span between preparations of sections from each cohort of monkeys prompted gross comparison of mRNA degradation. Actin mRNA was detected with digoxigenin ISH in the same manner as previously described for other mRNAs (24, 28–30). Digoxigenin-labeled human β -actin probe (550bp) was used (Roche Applied Science, Indianapolis, IN). Extracting RNA from formalin fixed tissue was not reliable.

In situ hybridization (ISH) assay for KISS1

Construction of the KISS1 cDNA template, transformation of plasmids, incorporation of digoxigenin into the reverse transcribed riboprobe and details of the ISH assay for KISS1 were previously published (29, 31)

Stereological Analysis of KISS1

KISS1 neurones appeared in the ARC, which in primates, enlarges and extends into the Infundibular nucleus (INF). This morphology is illustrated in Figure 1. Three levels of the INF similar to sections 2, 3 and 4 in Figure 1, were analysed for each animal. The morphology of the KISS1 population in level 1 was inconsistent between animals and added more variance to the analysis.

Montages of the INF were created with the Marianas Stereology Workstation and Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO). All images were transferred to Image J (NIH). For each anatomical level, a square outline was placed over the area and the exact dimensions were recorded. The same size square was then used for all of the animals at that anatomical level. Image J software segmented 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 or boutons in the defined area. The same procedure was applied at each anatomical level. Therefore, for each section the following data were obtained: total area measured, cell or bouton number, positive pixel area, cell size in pixels and fraction of the total area that was positive. Further calculation yielded the average cell number, average positive pixel area, average cell size and average positive fraction of the 3 sections for each animal. The animal means were subjected to statistical analysis. KISS1 images were also captured with a Leica DFC290 camera mounted on a Leica brightfield microscope.

Immunohistochemistry (IHC) for serotonin, NE, and GnRH

The IHC protocol for serotonin has been previously described (16). Serotonin IHC was performed on the anterior hypothalamus of the same animals used for KISS1 ISH. This involved the use of the last remaining sections from the first cohort of non-stressed animals that had been mounted and frozen for ISH. For comparison, identically mounted and frozen sections from the stressed cohort 3 were used. The use of these sections required an antigen retrieval step at the beginning of the assay as previously described (32), and each anatomical level was executed in one assay that included all animals. This exhausted all of the sections from the first non-stressed cohort 1.

In further IHC assays, floating sections were used from non-stressed placebo-treated controls from cohort 2 for comparison to the stressed animals of cohort 3. Serotonin axon density was examined in the periaqueductal gray (PAG). Because of other questions at the time, the serotonin IHC assays on the PAG of the non-stressed placebo-treated animals were conducted earlier than the stressed animals. The immunostained sections were subjected to image analysis at the same time, but using assays conducted at different times precluded combined statistical analysis in 2-way ANOVA.

NE innervation of the hypothalamus was inferred from detection of dopamine beta hydroxylase (DBH), the final enzyme in the pathway for NE synthesis. The DBH IHC assay was conducted in the same manner as the serotonin IHC assay (16). The primary antibody to DBH (Immunostar, Hudson, WI; formerly Incstar) was used at a concentration of (1:4000). The secondary antibody was an anti-rabbit IgG conjugated to biotin (Vector Laboratories,

Burlingame, CA) and used at a concentration of (1:500). The assay was conducted on floating sections of non-stressed placebo-treated animals of cohort 2 and stressed animals of cohort 3.

Although we previously examined GnRH in the ME of the non-stressed animals (33), we endeavored to examine this parameter in non-stressed and stressed animals at the same time. Hypothalamic GnRH axon densities were compared in floating sections from non-stressed placebo-treated animals of cohort 2 and stressed animals of cohort 3 with the same antibody and protocol as previously described (33).

Stereological Analysis of Immunostaining

The sections were morphologically matched between animals using anatomical reference points such as the infundibular recess of the 3rd ventricle (IR), the arcuate nucleus (ARC), the median eminence complex (ME), and the mammillary bodies in the hypothalamus as well as the dorsal raphe complex in the midbrain. A Marianas Stereological workstation (Intelligent Imaging Innovations, Denver, CO) with Slidebook 5.0 (Olympus Imaging Systems, Center Valley, PA) was used for image capture. A montage of the entire area containing GnRH, DBH, or serotonin immunostaining was built by the workstation. The image was exported as a Tiff file and ImageJ (NIH) was used to crop and adjust the illumination to the same level for all sections. The area containing staining of interest was 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 obtained, and then with a filter, the bouton number was obtained.

Statistical Analysis of In Situ Hybridization and Immunohistochemistry signals

Three anatomical levels were examined for KISS1 mRNA at 250 μ m intervals through the infundibular ARC; 2 sections were examined for GnRH axon density at 250 μ m intervals in lateral areas at the rostral end of the Infundibular Recess (IR); 4 levels were examined for DBH axon density at 250 μ m intervals through the rostral ARC; 4 levels were examined for hypothalamic serotonin axon density at 250 μ m intervals in the rostral periventricular area, and 7 anatomical levels were examined for PAG serotonin axon density at 250 μ m intervals in sections containing the dorsal raphe nucleus. The section/level results were averaged, generating one average for each animal. The average of the individual animals in each group was subjected to statistical analysis, so the variance around the treatment group is due to animal-to-animal variation. Most data were compared with 2-way analysis of variance (ANOVA) followed by Bonferroni's post hoc pairwise comparison. The PAG serotonin axon density in the non-stressed and stressed animals was analyzed separately with a t-test due to performance of IHC assays at different times. 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.

LH and E Immunoassays

Radioimmunoassays (RIA) were performed on serum by the Endocrine Technology and Support Lab, Oregon National Primate Research Center (Beaverton, OR). Serum LH was determined using a double-antibody RIA procedure similar to that described by Niswender and Spies (34). The LH RIA kit was purchased from Dr. Albert Parlow (NHPP, Harbor-UCLA Medical Center, Los Angeles). This is a homologous cynomolgus macaque assay with cynomolgus LH (AFP-6936A) for both iodination and standards. The rabbit anti-cynomolgus LH, AFP-342994, was used at a final dilution of 1:972,973. The detection limit of the assay was 0.015 ng/tube. The intraassay variation was 6.6%. Interassay variation was less than 10%.

Oestradiol radioimmunoassay was performed on serum using a Roche Diagnostics Cobas e411 automatic clinical platform assay instrument. The assay has been validated for macaque plasma in comparison with traditional RIA. Within-assay coefficients of variation were less than 5% and between-assay coefficients of variation were less than 10%.

Results

The population of KISS1 neurones that has shown regulation in different paradigms is located in the arcuate (ARC) nucleus of the medial basal hypothalamus. In primates, the archetypical ARC extends caudally and enlarges within the anatomical region called the Infundibulum as shown in Figure 2. In primates, the combination of the two areas is called *either* the infundibular nucleus or the ARC nucleus (BrainMaps.org). Due to different aspects of this study, we needed to distinguish the anatomical areas. Therefore, the archetypical ARC will be referred to as the rostral ARC and the infundibular extension of the ARC will be referred to as the INF ARC. The photographs in Figure 2 are montages of the areas exhibiting KISS 1 neurones stained with digoxigenin-ISH. The montages are generated by the Marianas stereology workstation and Slidebook 5.0 software. At level 1, KISS1 neurones are evident in the rostral ARC bordering the bottom of the 3rd ventricle. Level 2 is approximately 500µm caudal to level 1 and the INF ARC is evident with KISS1 neurones on both sides of the Infundibular Recess (IR) of the 3rd ventricle. Level 3 is approximately 250µm caudal to level 2 and contains the widest expansion of the INF ARC. The entire INF ARC, IR and median eminence (ME) are located ventral to the emerging mammillary bodies in our plane of sectioning. In level 4, located approximately 250µm caudal to level 3, the INF ARC is still visible with a significant number of KISS1 neurones, but the recess of the 3rd ventricle has shortened and the mammillary bodies have migrated lower in the overall anatomy. As sectioning continues in the caudal direction, anatomically the Infundibulum and ME disappear and the mammillary bodies occupy the ventral area of the brain. In this study, 3 consistent sections within the region of levels 2–4 were chosen from each animal for KISS1 assay and analysis.

Representative sections stained with digoxigenin-ISH for KISS1 mRNA from each group of animals are shown in Figure 3. These photographs are montages generated by the Marianas stereology workstation and Slidebook 5.0 software. This type of image was utilized for the measurements obtained with Image J. The top photomicrographs are from one HSR animal and one SS animal that were euthanized in the absence of stress (cohort 1). The bottom

photomicrographs are from one HSR and one SS animal that were euthanized after 5 days of relocation and diet (cohort 3). There are very few KISS1 positive neurones in the HSR animal without stress. The SS animal has numerous KISS1 positive neurones even in the absence of stress. After 5 days of stress, both the HSR and SS animals have numerous KISS1 positive neurones. The insert in the top right panel shows KISS1 positive neurones at higher magnification with brightfield optics.

The quantitative analysis of KISS1 mRNA expression is shown in Figure 4. The top panels illustrate the average cell number and average positive pixel area while the bottom panels illustrate the average cell size and average fraction of the total area that was positive for KISS1. The SEM represents the animal-to-animal variation. In all parameters, there was a significant effect of stress (2-way ANOVA, $p=0.006$; $p=0.004$; $p=0.01$, and $p=0.005$, respectively). The HSR group exhibited a significant increase in KISS1 cell number, positive pixel area, cell size and positive area fraction with stress compared to HSR animals without stress (groups with the same letter are different, Bonferroni's post hoc test, $p<0.05$). No statistical difference was found between the SS animals with and without stress. The SS animals trended higher than the HSR animals in the absence of stress, but the animal-to-animal variation was large and prevented achievement of a statistical difference.

The GnRH axons were detected in the basal hypothalamus at the rostral end of the IR as shown in Figure 5, **top panels**. In the top left panel, a representative section is shown, which was immediately rostral to the beginning of the IR, and the box illustrates half of the area analyzed. For image analysis, the ME was excluded and only those axons in the neuropile were measured. Also, both sides of the hypothalamus were included in the boxed area. Figure 6 **panel A**, illustrates the quantitative analysis of GnRH axons. There was a significant difference in GnRH axon density due to stress (2-way ANOVA, $p=0.015$) and a trend toward an effect of sensitivity ($p=0.060$). Bonferroni's posthoc test detected a significant increase in GnRH axon density in SS+stress animals over SS-no stress animals ($p<0.05$).

The serum concentrations of luteinizing hormone (LH) and E were determined in samples obtained at necropsy and the results are illustrated in Figure 6, **panels B and C**. Although LH appeared to gradually increase across the groups, there was no significant difference in serum LH at the time of necropsy. Unfortunately, the number of animals per group is low and the variance is quite high. After the LH assay, serum was depleted in 2 animals prior to the E assay. There was a trend toward difference (2 way ANOVA) in E between the groups due to sensitivity ($p=0.083$), but no difference due to stress or interaction. More animals are needed to increase the power of these measurements.

To compare the expression of KISS1 (pixels) with downstream hormone secretion, correlational analyses were conducted. The one significant correlation related to a downstream target of KISS1 is shown in Figure 6, **panel D**. GnRH was significantly correlated with serum LH (panel **E**, $r^2=0.91$, $p=0.044$). However, there was no correlation between serum E and KISS1 (pixels), between E and GnRH (pixels), or between E and serum LH. There was no correlation between GnRH and KISS1, or between GnRH and the ratio of KISS1/E.

Potential upstream effectors of KISS1 expression were examined. The hypothalamic DBH immunostaining is shown in Figure 5 **middle panels**. On the left, DBH axons were prominent in the rostral ARC nucleus and the box outlines the area that was analyzed. As shown on the right, DBH axon density was robust in the rostral ARC nucleus. Figure 7 illustrates the density of DBH axons in the rostral ARC across the groups. The average positive pixel area and the average number of DBH positive boutons were subjected to analysis with 2-way ANOVA. There was a significant effect of stress on bouton number ($p=0.002$) and positive pixel area ($p=0.0008$). However, there was no effect of sensitivity or interaction. Significant posthoc pairwise differences were detected for bouton number and pixel area between 'no stress' and '+stress' in both sensitivity groups ($p<0.05$). In addition, KISS1 expression was significantly correlated with the ratio of DBH pixels/E ($r^2=0.90$, $p=0.052$).

Representative sections containing hypothalamic serotonin immunostaining are shown in Figure 5 **bottom panels**. For this evaluation, rostral hypothalamic sections were examined from the same animals used for KISS1 mRNA examination. The box outlines the area that was analyzed. As illustrated in the right panel, the serotonergic innervation to the rostral periventricular area was robust. Figure 8 **panel A** illustrates the average positive pixel area and the average number of serotonin positive boutons in the rostral hypothalamus. Both data sets were subjected to analysis with 2-way ANOVA. There was a significant effect of sensitivity ($p=0.03$) on serotonin positive pixel area, but there was no effect of stress. The number of serotonin boutons showed the same pattern as the serotonin positive pixels, but did not differ significantly between the groups ($p=0.13$).

The periaqueductal gray (PAG) serotonin immunostaining and analysis are shown in Figure 8 **panels B and C**. PAG serotonin fibers arise in the dorsal and median raphe and innervate all areas of the forebrain, including the rostral periventricular area. As illustrated, the serotonergic innervation through the PAG area was robust. The average serotonin positive pixel area and the average number of serotonin positive boutons were subjected to analysis. Since the non-stressed and stressed animals were examined at different times, the data could not be directly compared with 2-way ANOVA. Instead, the t-test was used to compare HSR and SS animals independently in the presence and absence of stress. Although the data may be limited, it indicates that HSR animals have significantly higher serotonin boutons and positive pixels than SS animals regardless of stress, and similar to the hypothalamic innervation to which it contributes. Of note, the ratio of hypothalamic 5HT pixels/DBH pixels trended toward correlation with serum LH ($r^2=0.84$, $p=0.07$, not shown). However, serotonin alone or in a ratio with DBH or E did not correlate with other potential downstream targets.

Following ISH for actin, there was no obvious RNA degradation between the sections of hypothalamic tissue from the non-stressed animals compared to the stressed animals.

Discussion

Exposure to stressful stimuli can lead to a variety of secondary diseases such as anxiety, depression, cardiovascular disease, and immune hyperactivity (35, 36). Reproductive

dysfunction has been recently added to this growing list of stress-related disorders (10, 37). FHA is a disorder that occurs in females with no identifiable organic cause who tend to score higher than average on psychometric tests for stress; who diet but do not qualify for anorexia or other eating disorders; who exercise regularly; and who have no menstrual cycles for over 6 months (38–40). It is clear that some individuals are very sensitive to stressors, while others are stress resilient. Approximately 15% of women in the United States have some form of infertility and about 30% of female infertility is due to FHA or Stress-Induced Amenorrhea (41). Effective stress management and removal of metabolic stresses result in the restoration of fertility in most patients (42–44).

Hypothalamic GnRH has long been accepted as the master manipulator of reproductive function and several lines of evidence indicate that kisspeptin stimulates GnRH neurones. Administration of kisspeptin induces a large release of GnRH into portal blood in several species including humans (45–48); and a GnRH antagonist completely blocks the ability of kisspeptin to induce LH secretion in rodents, sheep and humans (49–51). Moreover, lack of the kisspeptin receptor, GPR54, is present in a consanguineous family with hypogonadotropic hypogonadism (IHH) (52).

One of the most potent regulators of KISS1 gene expression, and by inference kisspeptin production, is oestradiol (E). E markedly reduced KISS1 expression in the ARC in several species, constituting a negative feedback loop (5, 29, 53). E also stimulates KISS1 expression in kisspeptin neurones of the anteroventral periventricular nucleus (AVPV) in rodents or preoptic area (POA) in sheep, which may promote the LH surge (5, 54, 55). It has been suggested that this role is performed by a subpopulation of neurones in the ARC nuclei of guinea pigs, primates and humans (5, 56–58). In addition, E is essential for the developmental expression of KISS1 in the mouse (59). This study was performed on day 5 of the follicular phase and focuses exclusively on the population of KISS1 neurones that are governed by negative feedback of E.

We originally hypothesized that kisspeptin and KISS1 expression might play a role in the etiology of FHA. However, it should be noted that the majority of our neuroendocrine studies have used animals in the follicular phase and these measurements may be completely unrelated to events in the ovulatory phase. During the follicular phase, KISS1 is under negative feedback by E (5–7). This was supported by KISS1 expression in the non-stressed groups in which E was inversely correlated with KISS1 expression. However, KISS1 expression was increased by stress and the inhibitory regulation by E in the HSR group was absent in the presence of stress.

Downstream, KISS1 mRNA expression did not correlate with GnRH axon density, follicular serum E, follicular LH pulse frequency (27), or follicular serum LH when expression was examined in the presence and absence of stress. The best correlation, though not significant, was between KISS1 and the ratio of two downstream factors, GnRH/E, although what this means is far from clear (data not shown). Nonetheless, GnRH or GnRH/E correlated with serum LH with similar r^2 values. This data hints that E action at the level of the pituitary may be minor during negative feedback. Our data from the follicular phase is not comparable to data from Knobil and colleagues who showed that E could induce an LH surge

by direct pituitary action with constant intermittent GnRH replacement in positive feedback (60).

To examine the notion that stress information was conveyed to KISS1 neurones by potential upstream neurotransmitters, we examined the norepinephrine (NE) innervation of the rostral ARC nucleus using DBH as a marker. The NE innervation of the ARC has been suggested as a prime determinant of the LH surge and ovulation, and it is thought to derive from the nucleus of the tractus solitarius (61). DBH trended higher in SS than HSR animals in the absence of stress, and both groups exhibited a significant increase in DBH axon density with 5 days of moderate stress. The density of DBH axons in the rostral ARC nucleus as a single factor strongly trended toward correlation with KISS1 expression. However, the ratio of DBH/E significantly correlated with KISS1 expression. The concept of E negative feedback and stress-induced NE stimulation appears to best describe KISS1 expression in the presence and absence of stress.

Based upon our data and other studies (16, 62–65), it appears that stress increases NE; and we hypothesize that KISS1 is stimulated by NE with stress. Furthermore, the data suggest that stress-induced NE can override the inhibitory effect of E. These concepts are illustrated in Figure 9 for each group. The HSR-no stress group has robust early follicular phase E concentrations and little NE stimulation. Therefore, E inhibits KISS1 expression. The SS-no stress group has lower E and little NE stimulation. Hence, there is less inhibition of KISS1 by E and KISS1 expression is elevated. The HSR+stress group has robust early follicular phase E concentrations, but NE is elevated by stress. In this scenario, NE stimulation overrides E inhibition and KISS1 expression is elevated. In the SS+stress group, E is lower, providing less inhibitory tone, and NE is elevated by stress. Thus, NE stimulates KISS1 expression.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of NE, and the pattern of TH regulation detected in the dendritic halo of the locus ceruleus was identical to the pattern of DBH staining in the hypothalamus (16). Moreover, locus ceruleus NE is elevated by stress and this nucleus serves as an important gateway for transduction of stress (65, 66). Altogether, our limited observations, suggest that KISS1 mRNA is expressed in HSR and SS animals in the presence and absence of stress in a manner similar to NE produced in the locus ceruleus and detected in the hypothalamus, combined with negative feedback from E.

The concept that NE from the nucleus of the tractus solitarius (NTS) plays a role in stimulating the LH surge (61) is compatible with our suggestion that NE from the locus ceruleus responds to stress and also impacts reproduction. In addition, locus ceruleus tyrosine hydroxylase was regulated by ovulatory surge concentrations of E; and the NE neurons were reported to express ERalpha and ERbeta with ISH (67). However, ERalpha was not detected with IHC (68). As suggested in this study and previous studies, NE appears to be stimulatory to the HPG axis whether it is from the locus ceruleus (67) or the NTS (55). The effect of stress on both of these regions in the follicular and ovulatory phase would be informative. The data shown here in which KISS1-GnRH-LH are elevated by stress in the

follicular phase raises the question of whether, or how, such events in the follicular phase could precondition events in the ovulatory phase.

The other upstream factor examined was serotonin. We examined the serotonin projection from the midbrain PAG to the rostral hypothalamic periventricular area. Serotonin is involved in the regulation of LH (69), and we reasoned that serotonin might act on kisspeptin neurones. In the hypothalamus, where non-stressed and stressed animals could be assayed at the same time, serotonin axon density was higher in HSR than SS animals regardless of stress. A similar pattern was observed in the PAG although absolute numbers cannot be directly compared. The same pattern was also observed in TPH2 gene expression in serotonin neurons of the dorsal raphe (17); that is 5 days of moderate stress had no detectable effect. Serotonin did not correlate with any other factor in this study, but a similar pattern of innervation was detected in the LC, raising the idea that serotonin may modulate stress-induced NE at other levels. The idea of multilevel, multifactorial regulation of GnRH concurs with the wide body of literature in neuroendocrine reproductive biology.

We previously found that SS animals have markedly lower releasable serotonin (70) and that the selective serotonin reuptake inhibitor or SSRI, *s*-citalopram (CIT) increased ovarian steroid production in SS animals (71). Together this data initially suggested that serotonin mediated the effect of stress on reproduction. However, since CIT did not alter serotonin related gene expression (24) it is reasonable to speculate that CIT elevates *extracellular* serotonin, which in turn acts to modulate NE (72, 73) via innervation of the locus ceruleus (12).

Perhaps of interest, KISS1 expression correlates with previous measurements of CRF (data not shown). CRF stimulates NE neurons in the locus ceruleus (65, 74), and the density of the CRF innervation to the locus ceruleus significantly correlated with KISS1 cell size. CRF is a factor in the inhibition of reproduction by stress in rodents (75–77). In a feed-forward fashion, NE from the locus ceruleus also stimulates CRF (14), which in turn may impact KISS1 and GnRH neurons (78–80). However, these correlations are preliminary and further examination of CRF expression and innervation in the hypothalamus will be informative. Moreover, correlations are a long way from fully understanding the interactions of these systems and how they respond to stress and govern reproduction. Obvious questions regarding the expression of adrenergic receptors on KISS1 neurons, the regulation of the locus ceruleus NE neurons by follicular or ovulatory concentrations of E in non-stressed and stressed conditions, and the precise role of NE in negative feedback need further exploration.

Our observation that stress increases KISS1 in primates differs from observations in rodents where down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r (GPR54), mRNA expression is associated with stress-induced *suppression* of luteinizing hormone secretion (81). Immune or inflammatory reactions also reduced kisspeptin and LH in rodents (82, 83). Ferin (84) suggested that reactions to stress in primates vary considerably depending on E. In ovariectomized rhesus monkeys with very low to undetectable E, intracerebroventricular (icv) infusion of IL-1 α stimulated the hypothalamo-pituitary-adrenal (HPA) axis and inhibited pulsatile LH. This inhibitory effect of IL-1 α on LH was prevented by co-

administration of a CRH antagonist. Reintroduction of early follicular phase E concentrations completely prevented HPA activation and the inhibition of LH by IL-1a. In contrast, with reintroduction of late follicular phase E concentrations, the HPA responded to IL-1a, but LH release was stimulated. The icv administration of IL-1a is not the same as psychosocial and metabolic stress, although it is likely that immune activation is present. We posit that imitation of early follicular phase E followed by icv administration of IL-1 might be comparable to our model with 5 days of moderate stress in the early follicular phase. Rhesus monkeys are highly stress resilient, so comparison to our HSR group may be apt. Indeed, in HSR animals there was no significant effect of 5-day stress on circadian function of the HPA axis (23) and no effect on pulsatile LH secretion (27) even though stress increased KISS1 expression.

This study, a previous study in macaques (25) and a study in women (85) indicated that KISS1-positive cells were larger in ovariectomized females compared to E-treated females. We suggest that the larger cells have more robust production of KISS1/kisspeptin. Also, recent work has shown that an immediate pulse of GnRH follows a pulse of kisspeptin in macaques (86). It is attractive to speculate that infusion of kisspeptin into animals of different sensitivity, with or without concurrent stress, would exhibit different responses to kisspeptin. Further work is needed along this line of reasoning.

In conclusion, KISS1 mRNA trended higher in SS animals than HSR animals without stress. After 5 days of moderate stress, KISS 1 mRNA was elevated in *both* HSR animals and SS animals, but downstream markers of reproduction, individually, did not follow KISS1 expression. Despite the greater than 2-fold increase in KISS1 expression with stress in the HSR animals, there was no effect on GnRH or LH. The ratio of DBH/E predicted KISS1 expression. The LC could be the source of the stress-induced NE innervation of the hypothalamus or stress may act in a similar fashion on NE neurons in the NTS during the follicular phase. The results shown herein were obtained after only 5 days of moderate metabolic and psychosocial stress. Different results may ensue with longer periods of this type of stress.

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List of Abbreviations

HSR	highly stress resilient
MSR	medium stress resilient
SS	stress sensitive

FHA	Functional Hypothalamic Amenorrhea
HPG	hypothalamic-gonadal axis
HPA	hypothalamic-adrenal axis
LH	luteinizing hormone
E	oestradiol
CNS	central nervous system
mRNA	messenger RNA
ISH	in situ hybridization
IHC	immunohistochemistry
ANOVA	analysis of variance
KISS1	gene coding for kisspeptin, which stimulates GnRH neurons
GnRH	gonadotropin releasing hormone
NE	norepinephrine
CRF	corticotropin releasing factor
5HT	serotonin
INF	infundibulum
ARC	arcuate nucleus of the hypothalamus
INF ARC	extension of the arcuate nucleus in the infundibulum of primates
3V	third ventricle of the brain
MBH	medial basal hypothalamus
ME	median eminence
IR	infundibular recess of the 3 rd ventricle
MB	mammillary bodies

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Figure 1.

Photomicrographs of 4 anatomical levels through the arcuate nucleus (ARC) and infundibulum (INF) illustrating KISS1 neurones detected with digoxigenin-ISH. In level 1, the 3rd ventricle was present bordered by the classical ARC morphology (rostral ARC). It was dorsal to the median eminence (ME) and exhibited KISS1-positive cells. In level 2, which was 500µm caudal to level 1, the infundibular recess (IR) of the 3rd ventricle has formed and was bordered by the arcuate nucleus (INF ARC). Numerous KISS1-positive cells were detected. Level 3, which was 250µm caudal to level 2, also exhibited numerous KISS1-positive cells. Level 4, which was 250µm caudal to level 3, illustrates the smaller IR, which became shorter until it finally disappeared. The INF ARC remained, though smaller, and still exhibited a significant population of KISS1-positive neurones. With further sectioning, the INF eventually disappeared as the mammillary nuclei occupied the bottom of the diencephalon (not shown). Levels 2, 3 and 4 were the most consistently represented in all of the animals and were selected for further quantification of KISS1 expression.

Figure 2.

Representative photomicrographs are shown of KISS1 neurones developed with digoxigenin-ISH in the INF ARC nucleus in one animal from each group; HSR-no stress, SS-no stress, HSR+stress and SS+stress. KISS1-positive neurones were sparse in the HSR-no stress group. More KISS1-positive neurones were detected in the SS-stress group, but there was significant animal-to-animal variation. KISS1-positive neurones significantly increased in HSR+stress compared to HSR-no stress. KISS1-positive cells were also numerous in the SS+stress animals. The insert in the upper right corner illustrates the appearance of KISS1 cells processed with digoxigenin-ISH at a higher magnification under brightfield optics.

KISS1

Figure 3.

Histogram charts show the results of the quantitative analysis of KISS1 mRNA expression in the INF ARC. Each parameter was obtained with 3 sections from each animal. There were 4, 5, 4 and 3 animals in the HSR no stress, HSR+stress, SS-no stress and SS+stress, respectively. There was a significant effect of stress on average cell number, average positive pixel area, cell size, and in the fraction of the total pixel area that was positive for KISS1 (2-way ANOVA, $p=0.006$; $p=0.004$; $p=0.01$, and $p=0.005$, respectively). The lower bar over the no-stress groups is different from the top bar over the stressed groups (*). The HSR group exhibited a significant increase in all KISS1 parameters with stress compared to HSR animals without stress (Bonferroni's post hoc test, $p<0.05$; columns with the same letter are different). The SS animals without stress already had marked expression of KISS1, so the increase in the presence of stress was not statistically significant. Therefore, no pairwise statistical difference was found between the SS animals with and without stress.

Figure 4.

Photomicrographs of GnRH, DBH and serotonin (5HT) immunostaining in the hypothalamus. Panels on the left contain low magnification montages obtained with the Marianas Workstation and Stereoinvestigator 5.0 software. The boxes illustrate the areas that were further analyzed with Image J. For GnRH, the box included the other side of the hypothalamus as well. The right panels illustrate higher magnification pictures of GnRH (top), DBH (middle) and serotonin (bottom) immunohistochemical staining obtained on a Leica brightfield microscope.

Figure 5.

Histograms depicting the regulation of GnRH, LH and E, which are downstream targets of KISS1/Kisspeptin in HSR and SS animals in the presence and absence of stress. The numbers of animals available for each analysis are shown with small numbers in each column. Data were analyzed with 2-way ANOVA (bars). The downstream targets of KISS1 expression were subjected to linear regression analysis. **A.** Histograms illustrating GnRH axon density in the rostral ARC and INF ARC. There was a significant increase in GnRH with stress in both HSR and SS groups (bars; * $p=0.015$) and a trend toward a difference with sensitivity ($p=0.06$). There was a significant posthoc increase in GnRH with stress in the SS group (* $p=0.05$). **B.** Histograms illustrating serum LH under deep anesthesia prior to necropsy. There was no statistical difference in serum LH across the groups. LH appears slightly higher in the SS animals and to increase with stress, but with a low n and high variance in each group, it is not possible to draw conclusions. **C.** Serum E concentrations under deep anesthesia prior to necropsy. There was a strong trend toward lower serum E in the SS animals compared to the HSR animals (bars; # $p=0.08$), but there was no effect of stress. **D.** The only significant correlation is shown in a line graph $\pm$ 95% confidence intervals. Linear regression analysis showed a significant correlation between GnRH and serum LH ($r^2=0.91$, $p=0.044$).

Figure 6.

Histograms illustrating DBH axon density in the rostral ARC nucleus. The numbers of animals available for each analysis are shown with small numbers in each column. **Top.** In the rostral ARC, there was a significant difference in DBH bouton number and in DBH positive pixel area in the presence and absence of stress (2-way ANOVA $p < 0.0001$ and $p = 0.0008$, respectively).

Posthoc analysis indicated that DBH axon pixels and boutons increased with stress in both HSR and SS groups ($p = 0.05$).

Bottom. KISS1 significantly correlated with the ratio of DBH/E ($r^2 = 0.90$, $p = 0.052$).

Figure 7.

Histograms illustrating serotonin axon density in the rostral periventricular area and in the PAG. The numbers of animals available for each analysis are shown with small numbers in each column. **A.** In the hypothalamus, there was a significant difference in serotonin positive pixel area between HSR and SS groups (2 way ANOVA, $p=0.03$). Bars over the HSR and SS groups are different (*). There was a similar pattern in serotonin bouton number, but it did not reach significance. There was no effect of stress. **B.** Serotonin axon density was examined in the periaqueductal gray (PAG) at 7 levels of the dorsal raphe nucleus. In the absence of stress, sections from placebo-control animals (cohort 2) were used. In the presence of stress, sections from animals in cohort 3 were used. However, the non-stressed animals were processed for serotonin IHC at a different time than the stressed animals. Therefore, the data could not be compared with a 2-way ANOVA. Instead, the results are shown in separate histograms with the Y-axes adjusted and individually analyzed with a t-test. In a manner similar to the hypothalamic

serotonin axon analysis, the HSR animals have a consistently higher number of boutons and positive pixel area than the SS animals. **C.** Photomicrograph of the PAG axon density in a representative HSR animal from the stressed cohort 3 is shown. For PAG data *p= 0.007, t-test; # p=0.06, t-test.

Figure 8.

Integration of the information presented in this study. In the HSR-no stress group, E secretion was normal for the early follicular phase, and NE was at maintenance levels. This combination produced inhibition of KISS1 by E and little stimulation by NE. In the SS-no stress group, E was lower and NE was at maintenance levels. The lower E resulted in an increase in KISS1 expression. In the HSR+stress group, E was normal for the early follicular phase, but NE was elevated due to stress. The elevated NE prevented the E-mediated inhibition of KISS1, which in turn, became elevated. In the SS+stress group, E was lower and NE was elevated by stress. NE stimulated KISS1 expression with lower E as well, but the difference in KISS1 expression between Low E/Low NE, High E/High NE and Low E/High NE is negligible.