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Hypothalamic Expression of *KISS1* and Gonadotropin Inhibitory Hormone Genes During the Menstrual Cycle of a Non-Human Primate[1]

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ABSTRACT

Kisspeptin, the product of the *KISS1* gene, stimulates gonadotropin-releasing hormone (GnRH) secretion; gonadotropin inhibitory hormone (GnIH), encoded by the RF-amide-related peptide (*RFRP*) or *NPVF* gene, inhibits the reproductive axis. In sheep, kisspeptin neurons are found in the lateral preoptic area (POA) and the arcuate nucleus (ARC) and may be important for initiating the preovulatory GnRH/luteinizing hormone (LH) surge. GnIH cells are located in the ovine dorsomedial hypothalamic nucleus (DMN) and paraventricular nucleus (PVN), with similar distribution in the primate. *KISS1* cells are found in the primate POA and ARC, but the function that kisspeptin and GnIH play in primates has not been elucidated. We examined *KISS1* and *NPVF* mRNA throughout the menstrual cycle of a female primate, rhesus macaque (*Macaca mulatta*), using in situ hybridization. *KISS1*-expressing cells were found in the POA and ARC, and *NPVF*-expressing cells were located in the PVN/DMN. *KISS1* expression in the caudal ARC and POA was higher in the late follicular phase of the cycle (just before the GnRH/LH surge) than in the luteal phase. *NPVF* expression was also higher in the late follicular phase. We ascertained whether kisspeptin and/or GnIH cells project to GnRH neurons in the primate. Close appositions of kisspeptin and GnIH fibers were found on GnRH neurons, with no change across the menstrual cycle. These data suggest a role for kisspeptin in the stimulation of GnRH cells before the preovulatory GnRH/LH surge in non-human primates. The role of GnIH is less clear, with paradoxical up-regulation of gene expression in the late follicular phase of the menstrual cycle.

gonadotropin-releasing hormone, KISS1, *kisspeptin, menstrual cycle, preovulatory surge,* RFRP

INTRODUCTION

Reproduction depends upon secretion of the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) from the brain, which stimulates the synthesis and release of the gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone) from the pituitary gland. The secretion of GnRH and gonadotropins is controlled by feedback effects of gonadal steroids. In females, tonic negative feedback effects of estrogen and progesterone prevail throughout most of the ovarian cycle. In the late follicular phase of the cycle, a neuroendocrine switch occurs, and a transient, estrogen-induced positive feedback effect causes the preovulatory surge in GnRH/LH [1]. The surge in LH secretion causes ovulation. Because GnRH neurons do not possess the requisite sex steroid receptors [2–4], feedback signals to these neurons rely on transmission through other steroid-receptive cells within the brain. Kisspeptin is an RF-amide peptide that appears to play a major role in transmission of steroid feedback signals to GnRH neurons [5, 6]. Other recent studies strongly suggest hypothalamic cells that produce gonadotropin inhibitory hormone (GnIH) may oppose the actions of kisspeptin and inhibit the reproductive system [7, 8].

The RF-amide neuropeptide family was first described in the bivalve mollusk by Price and Greenberg [9] in 1977 with the discovery of a cardioexcitatory peptide from the cerebral ganglia. Two recently discovered members of this peptide family, kisspeptin and GnIH, appear to be significant components of the neuroendocrine system that regulate GnRH secretion and reproductive function. Moreover, kisspeptin-producing neurons appear to be ideally placed to act as the conduit for sex steroid feedback control over GnRH neurons [5, 6]. Kisspeptins are the peptide product of the *KISS1* gene; kisspeptins stimulate GnRH secretion [10–12] and appear to be critical for reproductive function [13, 14]. The stimulatory effect of kisspeptin on GnRH secretion appears to be fundamental to generation of the preovulatory LH surge in mice, rats, and sheep [15–17]. In sheep, *KISS1* mRNA-expressing cells are located in the arcuate nucleus (ARC) and the dorsolateral region of the preoptic area (POA) [18, 19]. It appears that the former cell group is important for the negative feedback regulation of GnRH [19], and cells of both regions may be important for generation of the preovulatory LH surge [17]. Notably, direct input to GnRH neurons is from the kisspeptin cells in the POA, whereas kisspeptin cells in the ARC may regulate GnRH neurons via an interneuronal pathway [20].

In human and non-human primates, kisspeptin-immunoreactive (ir) and *KISS1* mRNA-expressing cells are localized to the ARC [12, 21, 22], which is an area thought to be important for both positive and negative regulation to GnRH in these species [23, 24]. *KISS1* expression in the rhesus monkey ARC appears to increase over pubertal development [12], and *KISS1* mRNA expression increases in cynomolgus monkeys after ovariectomy [21], further substantiating a role for ARC kisspeptin cells in the negative feedback regulation of GnRH secretion. Evidence also exists, however, for the involvement

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of cells in the POA in generation of the preovulatory surge in primates [25], and *KISS1* mRNA is expressed in this region [26]. Because earlier studies indicated a surge-generating mechanism may exist in the POA of the non-human primate [25], we sought to determine whether a population of kisspeptin cells is present in the primate POA and, if so, what functional role kisspeptin cells in this region play in the preovulatory GnRH/LH surge.

Gonadotropin inhibitory hormone was discovered in the brain of the Japanese quail [27], and similar peptides were subsequently identified in mammals [28–31]. The mammalian forms have been termed RF-amide-related peptides (RFRP) transcribed from the *NPVF* gene [32], but the original nomenclature can be applied to all species [8]. Despite mounting evidence for a role in the regulation of GnRH secretion, it is unclear if GnIH is an important regulator of mammalian reproduction. Mammalian GnIH (also termed RFRP-3) reduces plasma gonadotropin levels when administered intracerebroventricularly or peripherally to a range of species [28, 33–36] and, in sheep, appears to play a hypophysiotropic role, inhibiting gonadotropin synthesis and secretion at the level of the pituitary gland [29, 37]. GnIH inhibits the firing of a subset of GnRH neurons in mice [38, 39], and GnIH terminals appear to make close appositions to GnRH neurons in mice, rats, hamsters, and sheep [28, 33, 39, 40–42]. Using immunohistochemistry and in situ hybridization, GnIH cells have been found in the male rhesus monkey brain, located in the intermediate periventricular nucleus (IPe) [30]. This location may bear some homology to the dorsomedial hypothalamic nucleus (DMN)/paraventricular nucleus (PVN) population of GnIH cells seen in rodents and sheep [28, 29]. These data suggest GnIH may play a role in the regulation of GnRH secretion/action in primates, as it does in other vertebrates.

Given the importance of these two RF-amide neuropeptide systems in the control of reproduction, we hypothesized that both systems play a role in regulation of the preovulatory LH surge in the non-human primate. Accordingly, the present study had two objectives: first, to determine whether mRNA expression of *KISS1* and/or GnIH (*NPVF*) vary over the rhesus monkey menstrual cycle and, second, to determine whether neurons expressing these neuropeptides make close appositions to GnRH neurons.

MATERIALS AND METHODS

Animals

Spontaneously cycling female rhesus macaques (*Macaca mulatta*; age, 7–12 yr; body wt, 5.5–6.5 kg) were individually housed and maintained in quarters kept at temperatures between 21 and 25°C with a 12L:12D photoperiod in accordance with National Institutes of Health guidelines. Monkey chow (Ralston Purina) was provided twice daily, and fresh fruit was provided once daily. Fresh water was available ad libitum. Animals were killed humanely during three hormonally defined stages of the menstrual cycle: luteal phase (n = 3), early follicular phase (n = 3), and late follicular (periovulatory) phase (n = 4).

For brain fixation, animals were killed by intravenous injection with 25 mg/kg of sodium pentobarbital followed by exsanguination under deep anesthesia in accordance with the recommendation from the Panel on Euthanasia of the American Veterinary Medical Association. The brain was perfused through the aorta with 1 L of saline followed by 3 L of cold 4% paraformaldehyde in 0.1 M borate at pH 9.5. The cranial and upper cervical vertebrae bone structures were removed, and the brain was extracted. The hypothalamus was postfixed and cryoprotected in 4% paraformaldehyde containing 20% sucrose. The hypothalamus was subsequently sectioned at 20 μm and collected at a 1-in-10 series (i.e., sequential sections 200 μm apart). Sections were mounted onto poly-L-lysine-subbed slides (two or three sections per slide), dried in a vacuum overnight, and stored at −80°C in sealed slide boxes.

Menstrual Cycle Determination

Serum samples, collected three times a week from monkeys, were submitted to the Endocrine Services Laboratory at the Oregon National Primate Research Center for determination of serum estradiol and progesterone concentrations. Those monkeys showing a normal menstrual cycle were selected for daily steroid determination. On the day of brain perfusion (during the midluteal, midfollicular, or late follicular phase), a serum sample was examined in the early morning. Estradiol and progesterone results were obtained to confirm the stage of the menstrual cycle (i.e., estradiol levels of 60–120 pg/ml during the midfollicular phase and >200 pg/ml during the preovulatory phase, and progesterone levels of >3 ng/ml during the midluteal phase). At necropsy, a blood sample was collected again for estradiol or progesterone determination.

Serum concentrations of estradiol and progesterone were determined using a validated chemiluminescence-based automatic clinical platform (Roche Diagnostics Elecsys 2010) [43]. The assay sensitivity was 20 pg/ml for estradiol and 0.2 ng/ml for progesterone. The intra- and interassay variations are less than 10% for both assays. All quality-control samples and validations, provided by the company, were analyzed in each assay.

Radiolabeled cRNA Riboprobes

KISS1 *riboprobe*. A 311-base cDNA sequence of the human *KISS1* gene (bases 209–519 of GenBank accession no. NM_002256) was cloned as previously described [12]. The antisense primate *KISS1* riboprobe was transcribed from linearized plasmid containing the *KISS1* insert with SP6 polymerase (Promega Corp.) and [^{35}S]uridine 5-triphosphate (GE Healthcare Life Sciences) under a standard transcription protocol. The riboprobe was separated from unincorporated nucleotides on a Sephadex G-25 column.

NPVF *riboprobe*. A 460-base cDNA sequence of the ovine *NPVF* precursor (*RFRP*, bases 43–502 of GenBank accession no. NM_001127268) was cloned as previously described [29, 41]. We predicted an ovine riboprobe would hybridize to *M. mulatta NPVF* mRNA (GenBank accession no. NM_001033115) because of the 80% homology between ovine and *M. mulatta* cDNAs in the cloned region. The antisense *NPVF* riboprobe was transcribed from linearized plasmid with T7 polymerase and [^{35}S]uridine 5-triphosphate as stated above.

KISS1 *and* NPVF *In Situ Hybridization*

In situ hybridization for *KISS1* and *NPVF* mRNA was performed as previously described [19]. For *KISS1* analysis, sections through the POA and ARC were chosen from each animal. For the POA, three or four sequential sections per animal were examined, and for the ARC, one or two sections per animal, representing the rostral, mid, and caudal regions of the ARC, were examined. For *NPVF* analysis, sections through the PVN/DMN region were chosen (n = 4–6 sequential sections per animal). The slides were prepared for in situ hybridization [19], and radiolabeled (^{35}S) antisense *KISS1* or *NPVF* riboprobe was denatured and then diluted in hybridization buffer at a concentration of 5×10^6 cpm/ml with tRNA (0.5 mg/ml). The hybridization solution was applied to slides (120 μl/slide) at 53°C for 16 h, after which the slides were treated with RNase A, washed in reducing concentrations of standard saline citrate, and dehydrated. The slides were then dipped in Ilford K5 photographic emulsion (Ilford Imaging), stored in the dark at 4°C, and developed 7 days later. No signal was observed after the application of radiolabeled sense probes (data not shown). Image analysis was carried out under dark-field illumination, and cells were counted when silver grain density was greater than fivefold the background level. The number of silver grains over each *KISS1*/*NPVF* cell (a semiquantitative index of mRNA content per cell) was determined using grain counting software (ImagePro Plus; Media Cybernetics, Inc.). For each animal, the number of *KISS1* or *NPVF* mRNA cells per section and silver grains per cell was averaged to produce the mean ± SEM in each region.

Immunohistochemistry

Kisspeptin and GnRH double-label immunohistochemistry. Three sequential sections through the POA and three through the mediobasal hypothalamus (MBH) were chosen from each animal for analysis. Antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (twice for 5 min each time). A blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in 0.1 M Tris-buffered saline (TBS) was applied (2 h at room temperature). The sections were then incubated for 72 h at 4°C with a sheep polyclonal antibody against synthetic human kisspeptin-54 (GQ2; kindly supplied by Dr. Stephen Bloom, Imperial College London,

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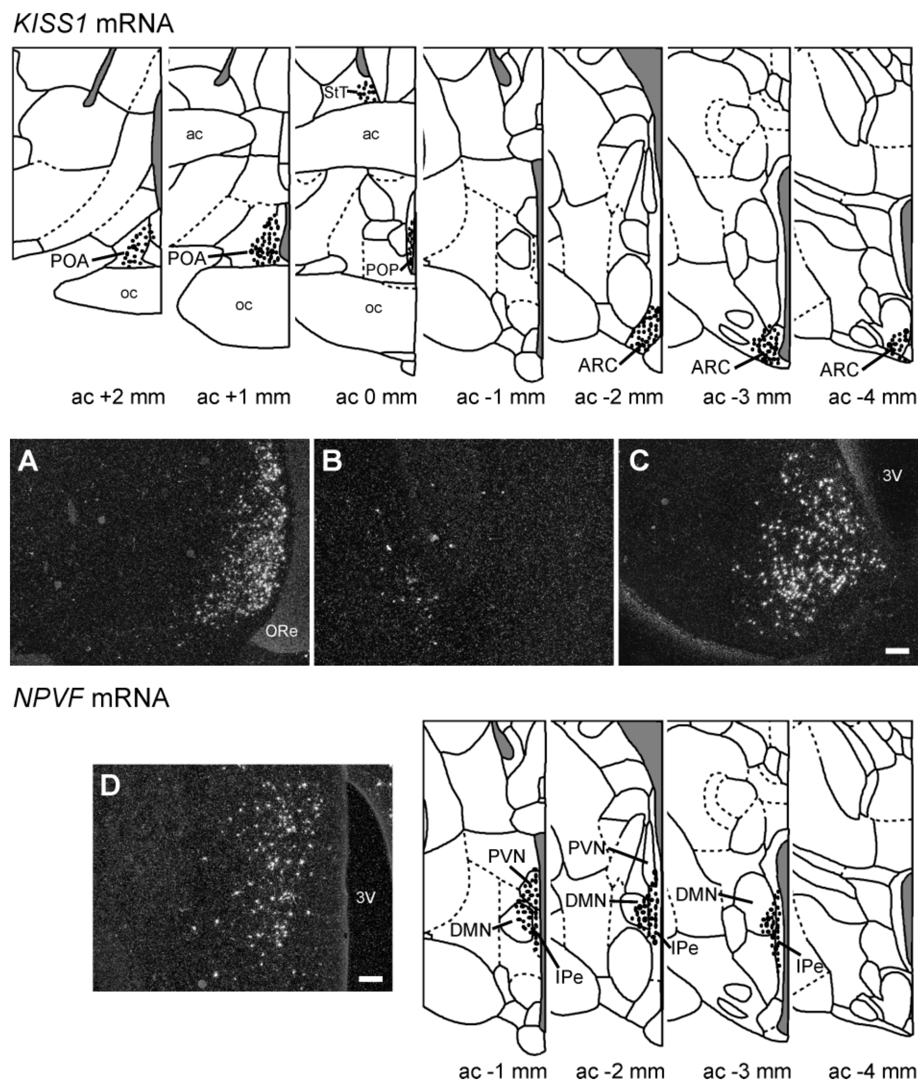

FIG. 1. Schematic drawings of coronal sections through the rhesus macaque brain showing the location of *KISS1* mRNA and *NPVF* mRNA (GnIH)-expressing cells (dots). Representative sections (modified from [45]) are 1 mm apart. Section serial numbers indicate the number of millimeters anterior (+) or posterior (−) from the complete anterior commissure (ac). **A**–**C**) Representative dark-field photomicrographs showing the distribution of *KISS1* mRNA-expressing cells in the POA (**A**), bed nucleus of the stria terminalis (**B**), and ARC (**C**). **D**) Representative dark-field photomicrographs showing the distribution of *NPVF* mRNA-expressing cells in the PVN/DMN. 3V, third ventricle; oc, optic chiasm; ORe, optic recess; POP, preoptic periventricular nucleus; StT, bed nucleus of the stria terminalis. Bars = 200 μm.

U.K.) previously used in monkeys [22] and a rabbit polyclonal antibody against GnRH (LR1; kindly supplied by Dr. Robert Benoit, Montreal General Hospital, Canada); both antibodies were at a dilution of 1:2000. The sections were washed in TBS, incubated with donkey anti-sheep Alexa 488 and goat anti-rabbit Alexa 546 (diluted 1:400; Molecular Probes, Inc.) for 2 h at room temperature, rinsed in TBS, and then counterstained with 0.3% Sudan Black B to minimize autofluorescence. Following rinses in TBS and then 0.1 M phosphate buffer, coverslips were applied using antifade mounting solution (DAKO).

GnIH and GnRH double-label immunohistochemistry. Three sequential sections through the POA and three through the MBH from each animal were chosen for analysis. Immunohistochemistry was performed as described above with the following exceptions: GnIH cells were visualized with a guinea pig polyclonal antibody against human GnIH (diluted 1:1000; human RFRP-3, VPNLPQRF-NH2; Antibodies Australia), which was previously shown to be specific in ovine tissue [42]. Goat anti-guinea pig Alexa 488 (diluted 1:400; Molecular Probes, Inc.) was used as a second antibody.

Image Analysis

The GnRH-ir cells were identified under fluorescent illumination, with a single observer counting the total number of GnRH cells and the number of GnRH cells with kisspeptin- or GnIH-ir terminal appositions. Putative contacts of kisspeptin and GnIH fibers on GnRH neurons were examined with a Zeiss Apotome microscope (Carl Zeiss, Inc.). Z-stacks of optical sections (1 μm, ×126 magnification) were captured through GnRH-ir neurons. Putative contacts were defined as apposition of terminals with soma or proximal dendrites when no pixelation occurred between the two objects. Using the Apotome system, Z-stacks were rotated to confirm the lack of pixelation between the objects when viewed in different planes. This method has been reported previously [42, 44]. For each animal, the percentage of "contacted" GnRH-ir cells in each region was averaged to produce the mean ± SEM.

Statistical Analysis

Data are presented as the mean ± SEM, and one-way ANOVA was used to determine the effect of menstrual cycle stage on *NPVF* mRNA expression as well as on estradiol and progesterone data. Two-way ANOVA was used to determine the effect of menstrual cycle stage on *KISS1* mRNA in the POA and rostral, mid, and caudal divisions of the ARC. Variation in the percentage of GnRH neurons with kisspeptin- or GnIH-ir terminal appositions was assessed by two-way ANOVA on arc-sine-transformed data, appropriate for the analysis of percentages. When significance was reached ($P < 0.05$), post hoc analysis of differences between means was performed by use of the least-significant-difference test.

RESULTS

Regulation of KISS1 and NPVF mRNA During the Menstrual Cycle

KISS1 mRNA-expressing cells were located in the POA and continued caudally to the preoptic periventricular nucleus (POP) (Fig. 1) [45]. *KISS1* cells were also abundant in the ARC (Fig. 1). A very small population of *KISS1*-expressing cells was also seen in the bed nucleus of the stria terminalis (Fig. 1), although no effect of menstrual cycle stage was found in this region (data not shown). The number of *KISS1*-expressing cells

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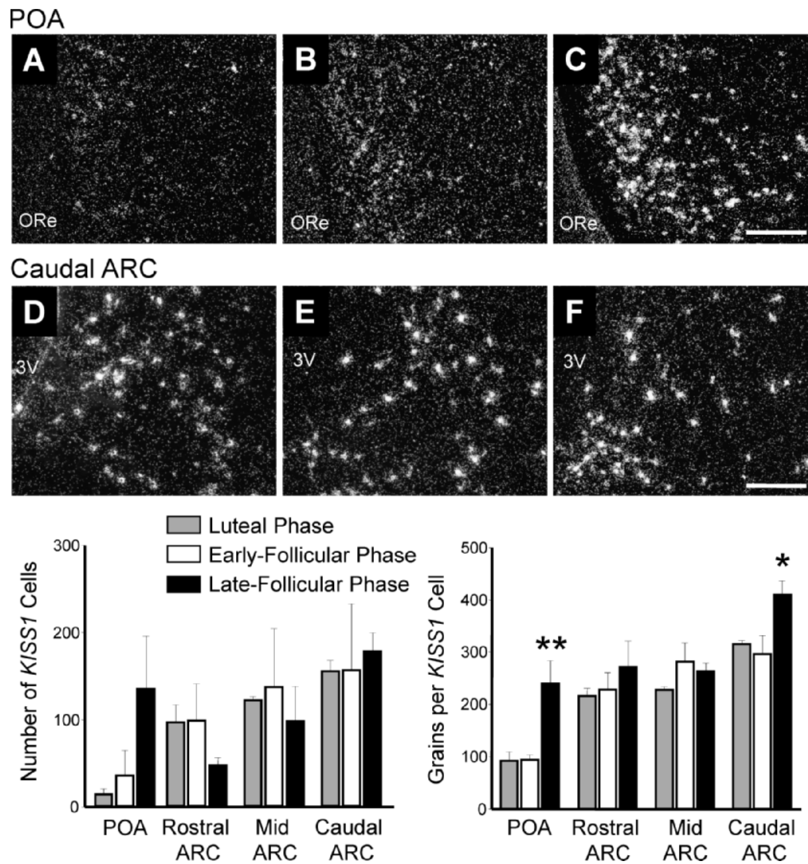

FIG. 2. Representative dark-field photomicrographs showing *KISS1* mRNA-expressing cells (as reflected by the presence of white clusters of silver grains) in the POA and ARC from rhesus macaques at the luteal phase (**A** and **D**), early follicular phase (**B** and **E**), and late follicular phase (**C** and **F**). Quantification of *KISS1* mRNA in the rostral, mid, and caudal ARC and the POA is shown. The number of *KISS1* mRNA-positive cells per section was unchanged over the cycle, but the number of grains per *KISS1* cell in the caudal ARC and POA was significantly greater in animals at the late follicular phase Values are presented as the mean ± SEM (n = 3–4 per group). 3V, third ventricle; ME, median eminence; ORe, optic recess. Bars = 200 μm. *$P < 0.05$, **$P < 0.01$.

in the POA and the ARC did not vary across the menstrual cycle (Fig. 2), although a nonsignificant trend for an increase in *KISS1* cells was noted in the POA. In the POA, the *KISS1* mRNA expression per cell was 2.5-fold higher ($P < 0.01$) during the late follicular phase of the menstrual cycle (239 ± 45 grains/cell) than in the luteal phase (92 ± 17 grains/cell) and early follicular phase (94 ± 9 grains/cell) (Fig. 2). In the caudal ARC, *KISS1* mRNA expression per cell was higher ($P < 0.05$) during the late follicular phase of the menstrual cycle (410 ± 26 grains/cell), being 38% higher than in the early follicular phase (296 ± 35 grains/cell) and 30% higher than in the luteal phase (315 ± 7 grains/cell; both $P < 0.05$) (Fig. 2). No change in *KISS1* mRNA expression per cell was observed in the rostral or mid ARC (Fig. 2).

NPVF mRNA-expressing cells were located in the PVN and DMN and extended medially into the IPe (Fig. 1). The number of *NPVF* mRNA-expressing cells did not differ during the menstrual cycle (Fig. 3). *NPVF* mRNA expression per cell was significantly reduced during the luteal phase of the menstrual cycle (155 ± 27 grains/cell) compared to the early follicular phase (49% reduced, 303 ± 11 grains/cell) and late follicular phase (46% reduced, 286 ± 42 grains/cell) (Fig. 3).

Kisspeptin- and GnIH-ir Terminal Appositions to GnRH Neurons

Kisspeptin-ir fibers in close apposition to GnRH neurons were located in the POA and MBH (Fig. 4A). The percentage of GnRH neurons displaying kisspeptin terminal appositions was greater in the MBH than in the POA irrespective of menstrual cycle stage ($P < 0.05$). Across the menstrual cycle, the percentage of GnRH neurons with kisspeptin appositions was similar in both the POA and the MBH (Fig. 4A).

We observed GnIH-ir fibers in close apposition to GnRH neurons in cells of the POA and MBH (Fig. 4B). No difference was observed in the percentage of GnRH neurons with GnIH terminal appositions between the POA and MBH or during the menstrual cycle.

Kisspeptin and GnIH fibers were distributed throughout the MBH (data not shown), as previously described in this species [22, 30]. One exception to these previous reports was that GnIH fibers were scarce in the median eminence at all stages of the menstrual cycle (Fig. 5).

Plasma Estradiol and Progesterone Concentrations

Estradiol levels were below the detectable limit of the assay in luteal-phase animals (14 ± 9 pg/ml). During the late follicular phase, estradiol levels (258 ± 46 pg/ml) were higher ($P < 0.01$) than during the early follicular phase (65 ± 11 pg/ml). During the luteal phase, progesterone levels (2.8 ± 0.9 ng/ml) were higher ($P < 0.05$) than in the early follicular phase (0.2 ± 0.1 ng/ml) and the late follicular phase (0.6 ± 0.05 ng/ml).

DISCUSSION

We describe the expression of *KISS1* and *NPVF* mRNA in the female non-human primate brain during the menstrual cycle. Our data show *KISS1* gene expression is greatest in the ARC and POA during the late follicular phase of the menstrual cycle, immediately preceding the preovulatory LH surge. *NPVF* mRNA expression was lowest during the luteal phase of the cycle (higher during the follicular phase). Both kisspeptin- and GnIH-ir terminals were seen to come into close contact with GnRH neurons. Together, these observations suggest kisspeptin neurons are involved as central processors for the

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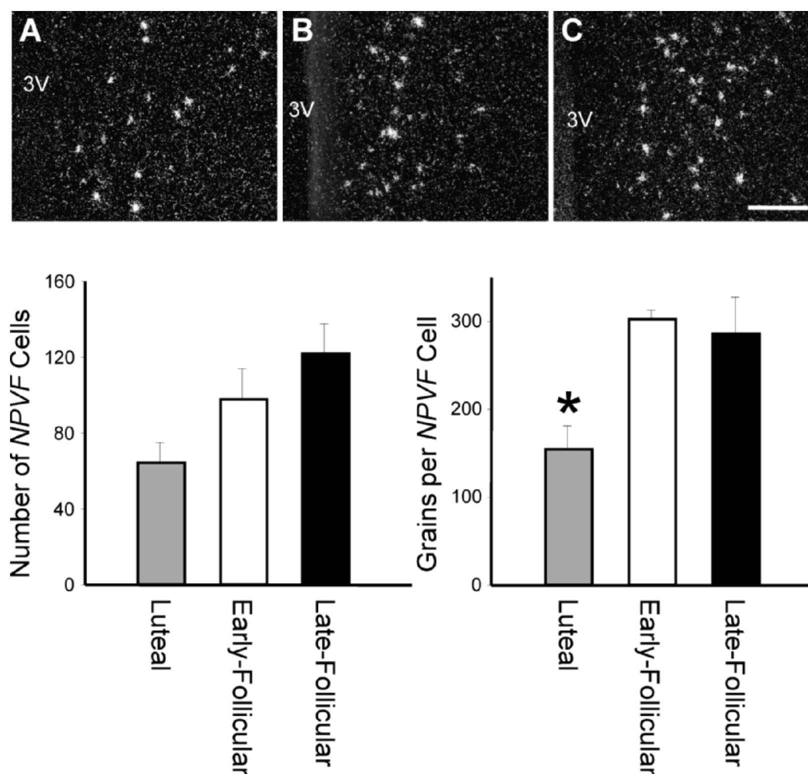

FIG. 3. Representative dark-field photomicrographs showing *NPVF* mRNA-expressing cells (GnIH; as reflected by the presence of white clusters of silver grains) in the PVN/DMN region from rhesus macaques at the luteal phase (**A**), early follicular phase (**B**), and late follicular phase (**C**). Quantification of *NPVF* mRNA shows the number of *NPVF* mRNA-positive cells per section was unchanged over the cycle, but the number of grains per *NPVF* cell was significantly reduced in luteal-phase animals. Values are presented as the mean ± SEM (n = 3–4 per group). 3V, third ventricle. Bars = 200 μm. *$P < 0.05$.

preovulatory GnRH/LH surge in the non-human primate. The role GnIH neurons play in this species is yet to be fully determined.

In a number of species, kisspeptin cells are ideally placed to act as the interneuronal link connecting levels of sex steroids to GnRH feedback regulation [5, 6]. Moreover, the acute positive feedback effects of estradiol to induce the preovulatory GnRH/LH surge appear to be transmitted (at least in part) by kisspeptin cells. In mice and rats, *Kiss1* mRNA expression is increased in the anteroventral periventricular nucleus (AVPV) by estradiol treatment, and this area of the brain is thought to be important for generation of the LH surge in these species [46–49]. Therefore, it is not surprising that *Kiss1* expression and activation of kisspeptin neurons (as indicated by the induction of FOS) increases in the AVPV at the time of the LH surge [15, 50]. Interestingly, in this species, KISS1 expression in the ARC appears to decrease or remain constant during this time [15, 50]. Estradiol acts via estrogen receptor α in the AVPV to regulate *Kiss1* in mice [51], and recent data show this to be via a classical estrogen-response element-dependent pathway [52]. Importantly, kisspeptin/GPR54 signaling, presumably in the AVPV, is essential for the LH surge in mice [16]. In sheep, a clear species difference is apparent: The MBH region of the brain, and not the AVPV, is critical for the acute positive feedback effects of estradiol on GnRH secretion [53, 54]. Consistent with this, *KISS1* mRNA expression in the caudal region of the ARC is greatest in the late follicular phase of the estrous cycle (immediately before the LH surge) [17, 18]. Moreover, kisspeptin neurons in the mid and caudal ARC become transcriptionally activated in response to an estrogen stimulus that induces an LH surge [17]. The kisspeptin cells in this region also appear to mediate the negative feedback effects of gonadal steroids, being up-regulated with ovariectomy and down-regulated with chronic estrogen replacement [19]. Interestingly, our recent data further suggest that, in addition to the *KISS1* gene expression in the ARC, *KISS1* gene expression in the ovine POA is up-regulated immediately before the LH surge [17], which may indicate similarities to the rodent species. Thus, we have proposed that both the ARC and the POA kisspeptin cells are involved in generation of the preovulatory LH surge in the ovine species.

The present results indicate the distribution of kisspeptin cells and changes in *KISS1* mRNA expression across the menstrual cycle in the female rhesus monkey are similar to that in the sheep. Moreover, we describe the distribution of kisspeptin cells in the POA of the non-human primate brain, which to our knowledge has not been described previously. It is possible a similar population of preoptic kisspeptin cells exists in the human brain [21], but this has not been scrutinized. *KISS1* expression in cells of the caudal ARC and POA was increased during the late follicular phase of the menstrual cycle. This indicates the likely involvement of both these populations of kisspeptin cells in generation of the estrogen positive feedback preovulatory LH surge in the primate. These findings in the non-human primate POA may be synonymous with those reported in the rodent AVPV, but definition of a precise role for these rostral kisspeptin cells may be problematic. Classic studies in rhesus monkeys where the MBH was surgically isolated from the rest of the brain show no interference in estrogen negative or positive feedback [23, 24, 55]. Such data potentially eliminate a critical role for the POA population of kisspeptin cells in generation of the preovulatory LH surge, but an active role cannot be ruled out. Thus, bilateral lesions to the POA in monkeys compromised the LH surge and ovulation [25]. Similar results were also noted after separation of the anterior hypothalamus and MBH [56], although blockade of LH surges and anovulation were only apparent in the short term (LH surges spontaneously resumed 120–210 days postoperatively). It is possible the anterior hypothalamic nuclei (potentially kisspeptin cells in the POA) do play a role in controlling the menstrual cycle of non-human primates but may not be essential.

Recent data in humans and non-human primates suggest *KISS1* expression in the ARC plays a role in estrogen negative

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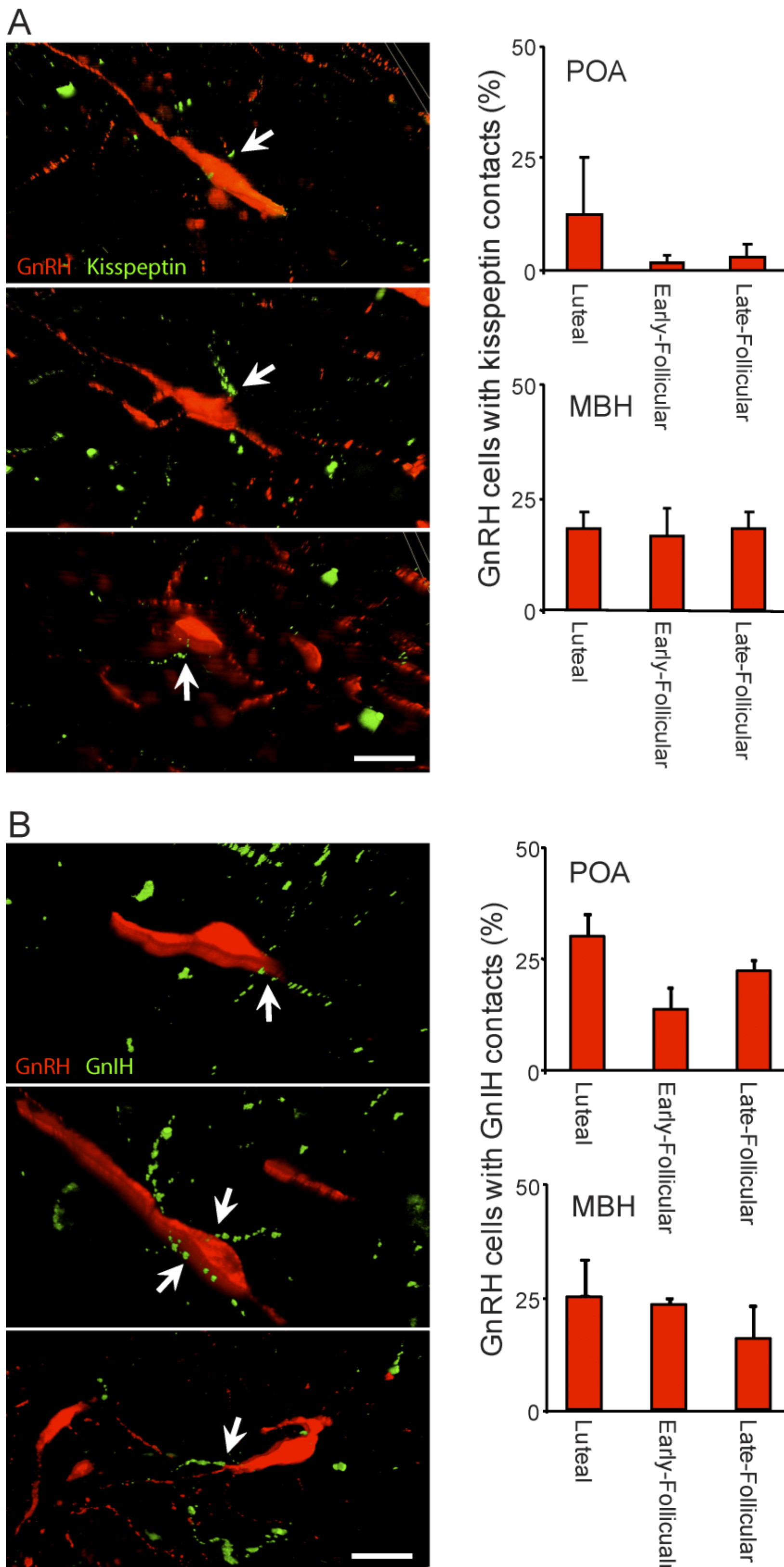

FIG. 4. Z-stack apotome analysis showing putative input to GnRH neurons from kisspeptin and GnIH terminals. The images show examples of GnRH neurons (red) that have kisspeptin fibers (**A**) or GnIH fibers (**B**; green) in close apposition. The percentage of GnRH neurons with kisspeptin or GnIH appositions was similar over the menstrual cycle in the POA and MBH. Values are presented as the mean ± SEM (n = 3–4 per group). Bars = 20 μm.

feedback [21]. We now show these cells may participate in the positive feedback phenomenon. Thus, as in the ovine species [17–19, 41], kisspeptin cells located in the ARC of the non-human primate appear to be involved in estrogen positive feedback regulation of GnRH, yet also play a role in chronic estrogen negative feedback regulation. Discrete regions of the ARC may be able to distinguish different estrogen stimuli and transmit negative or positive regulation of GnRH secretion. Alternatively, the same kisspeptin cells could respond to both negative and positive feedback stimuli, possibly involving classical and nonclassical ER pathways, as recently reported in the mouse [52]. In this regard, it is pertinent to note that acute

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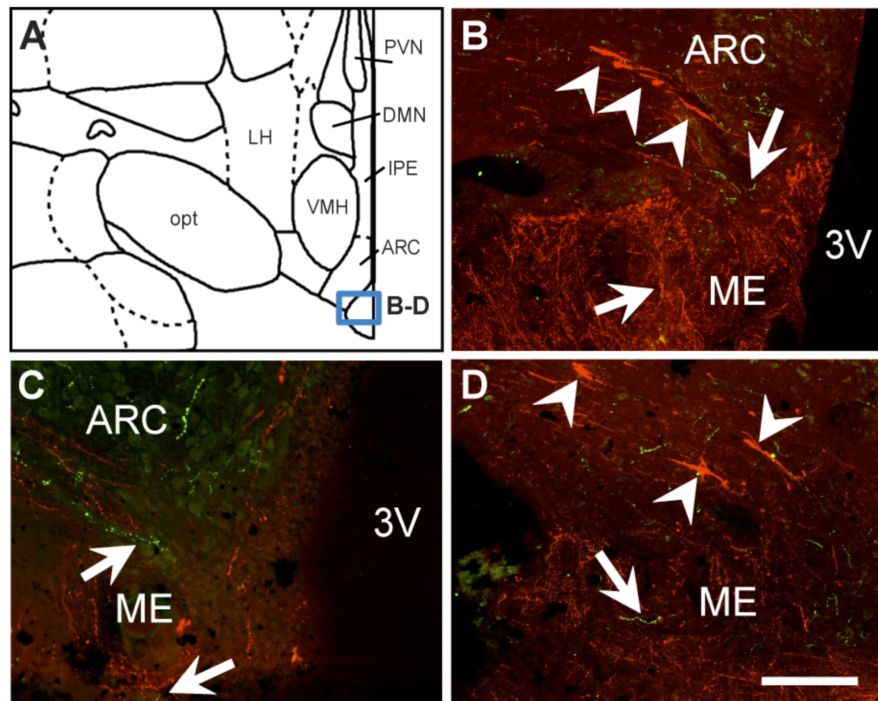

FIG. 5. Localization of GnIH-ir terminals in the median eminence of the rhesus macaque. Fluorescence photomicrographs show few GnIH fibers (green) located in the median eminence, where an abundance of GnRH fibers is noted (red). Arrows indicate GnIH fibers; arrowheads indicate GnRH neurons in the MBH. Box in **A** demonstrates the approximate position of photomicrographs **B**–**D**. 3V, third ventricle; ARC, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; LH, lateral hypothalamus; ME, median eminence; opt, optic tract; VMH, ventromedial hypothalamus. Bars = 100 μm.

(surge-inducing) estradiol treatment induced Fos expression in the vast majority of caudal ARC kisspeptin cells in sheep, whereas after ovariectomy, approximately half of the same kisspeptin cells had induced Fos expression [17]. Thus, it is probable some kisspeptin cells are responding to both positive and negative feedback signals.

Mammalian GnIH (*RFRP*) was first identified in the human, bovine, rat, and mouse [32] and was later characterized as the mammalian homolog to the avian GnIH [28]. GnIH inhibits gonadotropin secretion in hamsters [28], rats [33], and sheep [29, 37]. In the female rhesus monkey, we observed *NPVF* mRNA-expressing cells in the DMN and PVN, and this population appeared to extend medially into the IPe. In a recent study, GnIH (*NPVF*)-expressing cells were observed only in the IPe of the male rhesus monkey brain [30]. We believe this population of GnIH cells in males to be homologous to the one we describe in females. It is possible that disparities may relate to sex differences in GnIH expression in the rhesus monkey. In females, we show lower *NPVF* mRNA expression during the luteal phase than in the follicular phase of the menstrual cycle. Given the proposed role of GnIH in mammalian reproduction, especially in relation to the LH surge (in hamsters) [57], this result was unexpected. Functional studies are warranted to decipher the precise role of GnIH for various reasons. In rats, gonadal steroids do not appear to affect GnIH expression [58]. In sheep, similar results are apparent, because no change in *NPVF* mRNA expression occurs between breeding seasons or with ovariectomy and estradiol replacement [41]. However, changes in GnIH protein levels have been noted [41]. This inconsistency may relate to the nature of the *NPVF* mRNA, which may be rapidly degraded, or the *NPVF* riboprobe, which spans the 460-nucleotide ovine *NPVF* precursor. It is possible that posttranslational proteolytic cleavage of the GnIH preprotein may be regulated, leading to specific control of mature GnIH peptides.

In mice, GnIH directly inhibits the activity of GnRH neurons [38, 39], and kisspeptin stimulates their activity [59, 60]. Supporting this, GnIH- and kisspeptin-ir varicose fibers make close appositions to GnRH neurons in the mouse [39, 61], rat [33, 62], hamster [28], sheep [41, 42], and male rhesus monkey [22, 30]. We confirm these findings in female rhesus monkeys. Our previous data show GnIH and kisspeptin appositions to GnRH neurons vary with season in sheep, with GnIH appositions being fewer and kisspeptin appositions greater during the breeding season than during the nonbreeding anestrous season [41]. In the female rhesus monkey, both GnIH and kisspeptin appositions to GnRH neurons were unchanged over the menstrual cycle, indicating that involvement of these neuropeptides in the preovulatory LH surge is unlikely to occur at this specific level. In the non-human primate, GnIH-ir projections were also seen in the bed nucleus of the stria terminalis, habenular nucleus, and the PVN of the hypothalamus [30]. In the hypothalamus, GnIH fibers were mostly isolated to midline structures, such as the POA, PVN, ARC, and the DMN [30], and our data showed a similar distribution (data not shown). In the ovine brain, GnIH cells also project to appetite-regulating cells in the hypothalamus, particularly those producing neuropeptide Y and melanocortins in the ARC, those producing orexin and melanin-concentrating hormone in the lateral hypothalamic area, orexin cells in the DMN, and corticotrophin-releasing hormone and oxytocin cells in the PVN [42]. This is consistent with the proposed role for GnIH in the regulation of food intake [33, 35, 63]. Whether the same is true for the non-human primate remains to be determined.

The GnIH-ir terminals have also been visualized in the neurosecretory zone of the median eminence in hamsters [28] and sheep [29, 64], but not in rats [65]. Thus, a hypophysiotropic role has been proposed for some species but argued for others. In male rhesus monkeys, GnIH-ir terminals are seen in the median eminence [30], but we observed far fewer fibers in the present study. This difference may again relate to sex differences in the non-human primate but may also relate to the different antibodies used for immunodetection of GnIH. Kisspeptin-ir terminals have recently been detailed in the male rhesus monkey. In that study, abundant kisspeptin fibers were observed in the median eminence, and kisspeptin fibers also made infrequent contacts to GnRH neurons in the MBH [22].

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Our data, obtained in the female rhesus monkey, are consistent with these observations. Alternatively, in the male rhesus monkey, kisspeptin-positive perikarya were only observed in the ARC [22], whereas we observed a population of *KISS1* mRNA-positive cells in the POA. Two reasons are possible for this discrepancy. First, the initial study was performed in castrated males. Castration has been shown to reduce *Kiss1* mRNA expression in AVPV of rodents [51, 66], and it may reduce expression in the primate POA. Second, sex differences may be apparent in the more rostral populations of kisspeptin neurons (females > males), as also shown in rats [67], mice [68], and sheep [69]. The anatomical origin of kisspeptin inputs to GnRH neurons is yet to be fully determined in any species. In mice, data indicate the AVPV population of kisspeptin neurons as being the population that projects to GnRH neuronal cell bodies [61]. Consistent with this, estrogen-sensitive neurons located in the AVPV provide one of the largest direct inputs to GnRH neurons [48]. In sheep, close contacts of kisspeptin fibers on GnRH neurons may also come from the more rostral kisspeptin neurons. Credence for this is gained by the observation that the kisspeptin neurons of the ovine ARC do not appear to project to GnRH neurons, but those of the POA do [20]. Potentially, the POA kisspeptin population may form part of an interneuronal pathway connecting kisspeptin cells in the ARC to GnRH neurons.

Overall, our data describe the distribution of *KISS1* mRNA- and *NPVF* mRNA-expressing cells in the brain of the female rhesus monkey over the menstrual cycle. We show that levels of *KISS1* mRNA expression in the POA and caudal ARC increase during the late follicular phase immediately before the preovulatory LH surge. In addition, kisspeptin-ir fibers were seen to make close appositions to GnRH neurons (although this did not change across the menstrual cycle). These data suggest that both POA and ARC kisspeptin neurons are involved in the preovulatory GnRH/LH surge of the non-human primate. *NPVF* mRNA expression in the DMN/PVN region appeared to decrease during the luteal phase of the menstrual cycle, and GnIH-ir terminals made close appositions to GnRH neurons. The precise role GnIH cells play in the primate is yet to be determined.

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