Circadian Control of Kisspeptin and a Gated GnRH Response Mediate the Preovulatory Luteinizing Hormone Surge

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In spontaneously ovulating rodents, the preovulatory LH surge is initiated on the day of proestrus by a timed, stimulatory signal originating from the circadian clock in the suprachiasmatic nucleus (SCN). The present studies explored whether kisspeptin is part of the essential neural circuit linking the SCN to the GnRH system to stimulate ovulation in Syrian hamsters (*Mesocricetus auratus*). Kisspeptin neurons exhibit an estrogen-dependent, daily pattern of cellular activity consistent with a role in the circadian control of the LH surge. The SCN targets kisspeptin neurons via vasopressinergic (AVP), but not vasoactive intestinal polypeptide-ergic, projections. Because AVP administration can only stimulate the LH surge during a restricted time of day, we examined the possibility that the response to AVP is gated at the level of kisspeptin and/or GnRH neurons. Kisspeptin and GnRH activation were assessed after the administration of AVP during the morning (when AVP is incapable of initiating the LH surge) and the afternoon (when AVP injections stimulate the LH surge). Kisspeptin, but not GnRH, cellular activity was up-regulated after morning injections of AVP, suggesting that time-dependent sensitivity to SCN signaling is gated within GnRH but not kisspeptin neurons. In support of this possibility, we found that the GnRH system exhibits pronounced daily changes in sensitivity to kisspeptin stimulation, with maximal sensitivity in the afternoon. Together these studies reveal a novel mechanism of ovulatory control with interactions among the circadian system, kisspeptin signaling, and a GnRH gating mechanism of control. **(*Endocrinology* 152: 595–606, 2011)**

Despite the established role of the circadian system in regulating ovulation across species (1–3), the precise neurochemical pathways by which a daily timing signal initiates the preovulatory LH surge remain to be fully elucidated. In mammals, the orchestration of circadian rhythms is controlled by a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (4, 5). Circadian rhythms are endogenously generated (6, 7) and synchronized to the external environment via direct neural projections from intrinsically photosensitive retinal ganglion cells to the circadian clock in the SCN (8). The SCN communicates to hypothalamic cell phenotypes driving reproductive function through extensive direct and indirect neural projections (2, 9, 10). On the day of proestrus, in most spontaneously ovulating rodent species, the preovulatory LH is initiated by the SCN late in the afternoon (11–14). Perturbations of SCN output signaling pathways or intrinsic clock activity lead to gross deficits in female rodent ovulatory function and fecundity (15–18).

Two SCN-derived neurochemical pathways have been implicated in the initiation of the LH surge. The first is a monosynaptic pathway whereby SCN-derived, vasoactive intestinal polypeptide (VIP) secreting neurons project directly to GnRH cells (19). GnRH neurons targeted by VIP

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Abbreviations: AVP, Arginine vasopressin; AVPV, anteroventral periventricular nucleus; DBB, diagonal band of Broca; ER, estrogen receptor; GABA, γ-aminobutyric acid; ir, immunoreactive; i.c.v., intracerebroventricular; MPOA, medial POA; MS, medial septum; OVX, ovariectomized; POA, preoptic area; RFRP, RFamide-related peptide; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal polypeptide; ZT, zeitgeber time.

express FOS around the time of the LH surge and antisense oligonucleotides directed against VIP attenuate and delay the LH surge in estradiol-treated animals (20, 21). Despite these corroborating lines of evidence, other studies indicate equivocal effects of exogenous VIP administration, with VIP inhibiting GnRH in some instances and playing an excitatory role in others (20, 22). Additionally, VIP cells contact only a small percentage of GnRH cells (~5–20%) across rodent species (9, 23), well below the percentage activated at the time of the surge (24). Importantly, GnRH neurons do not express estrogen receptor (ER)-α (25, 26), the ER subtype responsible for the positive feedback effects of estradiol on the LH surge (27). The fact that SCN-derived VIP projections cannot fully account for the LH surge suggests the existence of an additional mechanism(s) of circadian control that coordinate with timed VIP stimulation of the GnRH system.

The anteroventral periventricular nucleus (AVPV) is a critical neural locus for the initiation of the LH surge. Lesions of the AVPV eliminate estrous cyclicity (28). Furthermore, neurons within the AVPV project to GnRH cells and exhibit FOS expression coinciding with the timing of the LH surge (29). The SCN projects to the AVPV providing a neural pathway for temporally controlling cell populations driving GnRH activity (30, 31). SCN cells targeting the AVPV express vasopressin (AVP) (32, 33), and AVP injections produce surge-like LH levels in SCN-lesioned, ovariectomized (OVX), estradiol-treated rats (34). Finally, ERα-expressing neurons within the AVPV are direct targets of the SCN (33), potentially integrating circadian signals and positive feedback actions of estrogen.

Although abundant evidence indicates that the AVPV is necessary for circadian initiation of ovulation in rodents, the specific neural pathway(s) and cellular phenotype(s) involved in this process have not been fully characterized. The stimulatory neuropeptide, kisspeptin, provides an attractive target for further exploration. Kisspeptin and *Kiss1* the gene encoding kisspeptin peptide, are expressed in the AVPV across species (35–37) and play a significant role in positively regulating the reproductive axis (reviewed in Ref. 38). Exogenous kisspeptin administration potently induces LH release and up-regulates FOS expression in GnRH neurons (35, 39). A large percentage of kisspeptin-immunoreactive (ir) neurons in the AVPV express ERα and *Kiss1* mRNA is up-regulated by estradiol administration in OVX animals (40). Finally, *Kiss1* cells express FOS at the time of the LH surge in naturally cycling and OVX, estradiol-treated rats (41, 42). Together these results suggest that *Kiss1* neurons in the AVPV participate in estrogen-positive feedback and are positioned to receive circadian clock input.

An additional layer of complexity in exploring the role of the circadian system in LH surge initiation is that administration of SCN neuropeptides induces the surge only within a narrow time window (43), suggesting additional temporal control at SCN target loci. The means by which this timed gating mechanism controls responsiveness of the hypothalamic-pituitary-gonadal axis to SCN communication remains to be investigated. The gating of SCN information flow may be controlled within the AVPV, at the level of GnRH neurons, or a combination of both loci. The present studies explored the role of AVP and kisspeptin signaling in the timing of the LH surge using several approaches. First, we examined whether kisspeptin neurons in the AVPV are targets of vasopressinergic SCN input. Next, we asked whether the kisspeptin system exhibits a daily pattern of neuronal activity consistent with a role in ovulation and whether any emergent pattern is estrogen sensitive. Finally, we explored the possibility that this circuit is responsible for time-dependent sensitivity of the reproductive axis to SCN signaling by assessing whether: 1) kisspeptin cells within the AVPV respond in a time-dependent manner to AVP stimulation, 2) GnRH neurons display time-dependent sensitivity to kisspeptin signaling, or 3) both kisspeptin and the GnRH systems coordinate to gate the timed initiation of the LH surge. If time-dependent sensitivity to upstream circadian signaling is controlled at the level of the AVPV, then one would expect kisspeptin cells to exhibit daily changes in sensitivity to AVP stimulation. Alternatively, if the gating of control occurs within GnRH cells, then one would expect the GnRH system to display daily sensitivity in response to both AVP and kisspeptin administration.

Materials and Methods

Animals

Adult (>60 d of age), female LVG Syrian hamsters (*Mesocricetus auratus*) (n = 91) were used. Hamsters purchased from Charles River (Wilmington, MA) at 4–5 wk of age were housed in translucent propylene cages (48 × 27 × 20 cm) and provided with *ad libitum* access to food and water at all times. Animals were maintained in a colony room at 23 ± 1 C with a 24-h light: dark cycle (14 h light, 10 h dark) with lights on at 0700 h and lights off at 2100 h. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Examination of the circadian pattern of kisspeptin activation

To determine whether kisspeptin expression is coordinated with the timing of the LH surge, we examined the activational state of AVPV kisspeptin neurons over the course of the day in ovariectomized hamsters given empty implants or implants containing estradiol (44). Before ovariectomy, estrous cyclicity was

monitored by daily examination of vaginal discharge (45), and only females with regular 4-d estrous cycles were retained for study. To examine the pattern of kisspeptin cell activation independent of fluctuations in peripheral sex steroids, females were bilaterally OVX (n = 48) under isoflurane anesthesia. To determine whether daily changes in kisspeptin expression are estrogen dependent, animals were either treated with a SILASTIC capsule (Dow Corning Corp., Midland, MI; 10 mm length, 1.45 mm inner diameter, 1.93 mm outer diameter) containing powdered undiluted 17β-estradiol (n = 24) or an empty capsule (n = 24). In estrogen-implanted hamsters, this treatment generates estradiol concentrations comparable with those seen on the day of proestrus and results in daily LH surges at the same time each day, approximately 4 h before the onset of darkness (46). Two weeks after the ovariectomy and capsule implantation, hamsters were perfused (as described below) at either zeitgeber time (ZT) 7, 11, 13, and 16 (darkness onset at ZT 14) (n = 6/group) and brains were collected for histological analysis.

Examination of kisspeptin cells for SCN-derived input and receptor expression

To determine whether the SCN sends AVPergic or VIPergic projections to kisspeptin cells in the AVPV, adult Syrian hamsters (n = 5) were perfused on the day of proestrus at ZT 11. Brains were collected and stained for either kisspeptin and AVP (n = 5) or kisspeptin, and VIP using immunofluorescence (n = 5) and fiber contacts onto kisspeptin cells were evaluated as described below. To ensure that any fibers found within the AVPV contacting kisspeptin cell bodies originate from the SCN, five additional hamsters received bilateral electrolytic SCN lesions. Under deep anesthesia induced with a ketamine cocktail (21 mg ketamine, 2.4 mg xylazine, and 0.3 mg acepromazine per milliliter injected ip in a dose of 0.34 ml per 100 g body mass), the head was shaved and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujanga, CA) and the hamster prepared for aseptic surgery. Lesions were aimed at the following coordinates: 0.9 mm anterior to bregma, 0.3 mm lateral to midline, and 7.8 mm below dura (31). Bilateral radio frequency lesions were made by applying 25 mV for 15 sec using a Radionics model RFG-4A Research RF lesion generator (Radionics, Burlington, MA) and stainless steel electrodes insulated with Epoxylite (The Epoxylite Corp., Irvine, CA), excluding the tip (0.20 mm). Lesioned brains were collected 24 h after surgery after perfusion as described below. Finally, to examine the coexpression of the vasopressin receptor, V1a, every fourth section was double labeled immunohistochemically for kisspeptin peptide and V1a protein.

Examination of kisspeptin and GnRH activation after timed central administration of vasopressin

Adult hamsters (n = 24) were OVX and implanted with SILASTIC capsules (Dow Corning) containing powdered 17β-estradiol as described above. After a 1-wk recovery period, a unilateral guide cannula (6 mm; Plastics One, Roanoke, VA) was implanted, aimed at the lateral ventricle. Coordinates for implantation were 0.6 mm lateral and 1.5 mm posterior to bregma and 4.5 mm ventral from the surface of dura mater. To maintain patency, dummy cannulas were attached to guide cannulae after surgery. After a 1-wk recovery period, cannulae placements were confirmed by assessing drinking behavior in response to angiotensin II injections (10 ng in 5 μl sterile saline). Because AVP fibers, but not VIP fibers (see *Results*), targeted kisspeptin cells, we focused on the role of this peptide in stimulating the LH surge. AVP (n = 12) (2 ng per 5 μl) or saline vehicle (n = 12) was administered early in the day at ZT 1 (when AVP is ineffective at stimulating the GnRH system) and in the afternoon at ZT 11 (when AVP stimulates the LH surge). Hamsters were perfused 1 h after injection and brains were collected and double labeled for kisspeptin/FOS and GnRH/FOS using immunofluorescence as described below.

Examination of GnRH activation after timed kisspeptin administration

To determine whether the GnRH system responds in a time-sensitive manner to kisspeptin and whether the GnRH system requires the presence of estradiol for the response to exogenous kisspeptin, hamsters were OVX and treated with empty (n = 36) SILASTIC capsules (Dow Corning) or capsules containing estradiol (n = 36). One week after surgery, kisspeptin (kisspeptin-10 (mouse); Phoenix Pharmaceuticals, Burlingame, CA) was injected (ip; 2 or 4 nmol) at ZT 1 or ZT 11 and hamsters perfused 1 h after injection. Brains were collected and double labeled for GnRH/FOS as described below.

Perfusion and histology

For brain collection, hamsters were deeply anesthetized with sodium pentobarbital (200 mg/kg) and perfused transcardially with approximately 150 ml of 0.9% saline, followed by 300–400 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Double-labeled immunofluorescence was performed on every fourth, 40-μm coronal section. Sections were incubated for 48 h at 4 C with a rabbit polyclonal antikisspeptin-10 antiserum (1:2000; generated by J.D.M.), previously shown to bind with high affinity to kisspeptin neurons in the AVPV and arcuate nuclei and exhibit minimal cross-reactivity to related RFamide (Arg-Phe-NH_2) peptides (47, 48), or rabbit anti-FOS (1:50,000; Santa Cruz Biotechnology, Santa Cruz, CA) and normal goat serum diluted at 1:1000 with 0.1% phosphate buffer containing Triton X-100 for 48 h. After incubation in the first primary antibody, brains were incubated for 1 h in biotinylated goat antirabbit (1:300; Vector Laboratories, Burlingame, CA) and then in avidin biotin complex for 1 h. For kisspeptin or FOS, the signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (49). Cells were then labeled by using Cy-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) as the fluorophore. After labeling the primary antibody, the sections were incubated in either a rabbit anti-GnRH antiserum (LR-5; 1:20,000; a generous gift from Dr. R. Benoit, McGill University Health Centre, Montreal, Quebec), rabbit anti-FOS antibody (1:5,000; Santa Cruz Biotechnology), a guinea pig anti-AVP antibody (1:10,000; Santa Cruz Biotechnology), a rabbit anti-V1a (1:500; Santa Cruz Biotechnology), or a guinea pig anti-VIP antibody (1:500; Santa Cruz Biotechnology) with 0.1% phosphate buffer containing Triton X-100 for 48 h (49). The second primary antibody was labeled with CY-3 donkey antirabbit (GnRH, V1a, FOS; 1:200; Jackson ImmunoResearch Laboratories) or CY-3 anti-guinea pig (AVP, VIP; 1:200; Jackson ImmunoResearch Laboratories) as the secondary antibody/fluorophore.

Several control procedures were implemented to ensure the specificity of immunohistochemical labeling. First, all antibodies were preadsorbed with their respective ligands for 24 h before tissue application. This procedure eliminated staining in all

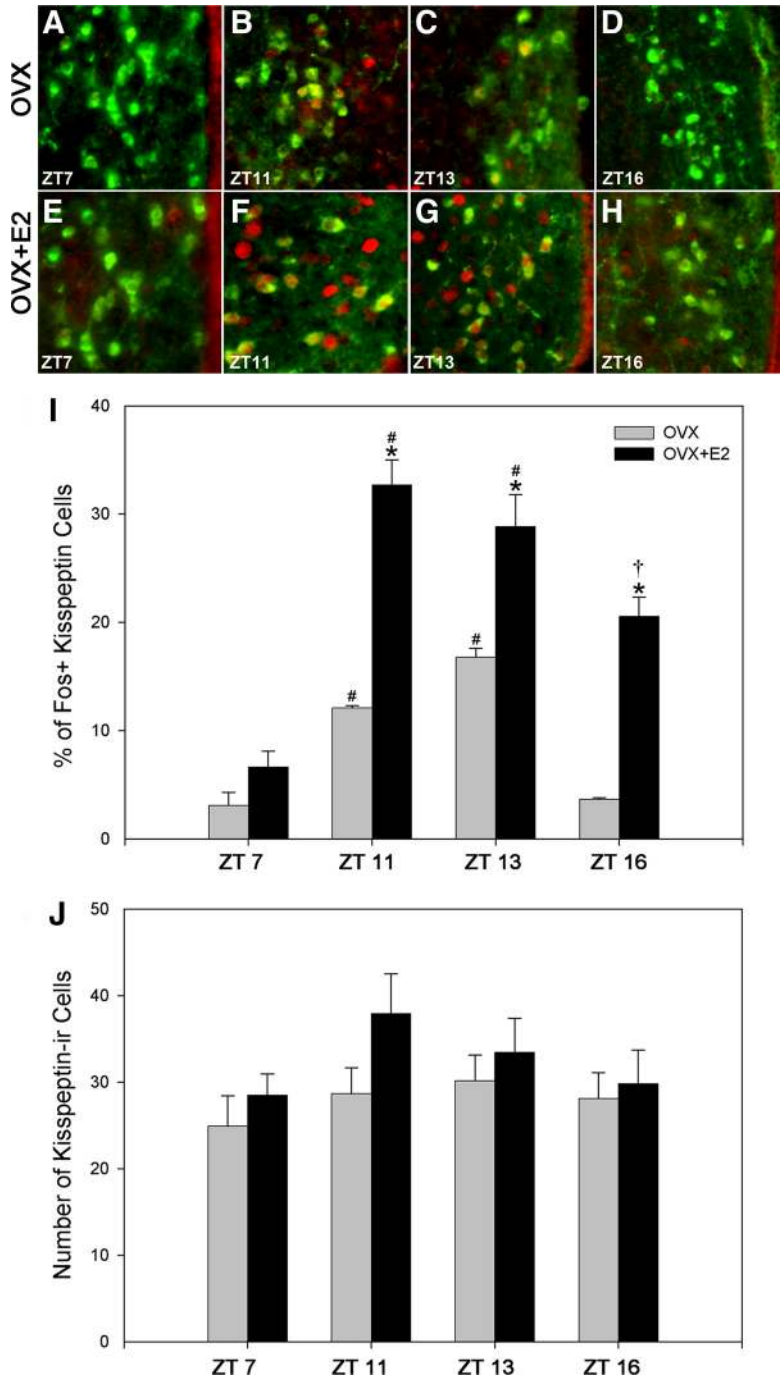

FIG. 1. AVPV kisspeptin cellular activity follows a daily pattern of expression coincident with the LH surge. The percentage of AVPV kisspeptin-ir cells expressing FOS increases in the afternoon and peaks at ZT 11, around the time of the LH surge, and decreases thereafter. The daily pattern of expression is robust in OVX+estradiol (E2) hamsters, with the magnitude of this daily pattern at each time point significantly attenuated in the absence of estradiol, excluding ZT 7. A–D, Low-power photomicrographs of kisspeptin-ir cells expressing FOS in OVX hamsters at ZT 7 (A), around the time of the GnRH surge at ZT 11 (B) and ZT 13 (C), and 2 h after lights out at ZT 16 (D). E–H, Low-power photomicrographs of kisspeptin-ir cells expressing FOS in OVX+E2 hamsters at ZT 7 (E), around the time of the GnRH surge at ZT 11 (F) and ZT 13 (G), and 2 h after lights out at ZT 16 (H). I, Mean (±SEM) percentage of kisspeptin-ir cells expressing FOS at various time points in OVX and OVX+E2 hamsters. J, Mean (±SEM) number of kisspeptin-ir cells at various time points in OVX and OVX+E2 females. #, Significantly greater than kisspeptin cells expressing FOS at ZT 7 and ZT 16 within the same hormonal treatment ($P < 0.05$); *, significantly greater than kisspeptin cells expressing FOS in OVX hamsters within the same time point ($P < 0.05$); †, significantly greater than kisspeptin cells expressing FOS at ZT 7 within the same hormonal treatment.

cases. Because kisspeptin is an RFamide peptide with a C terminus structure similar to that of RF-amide-related peptide (RFRP), the kisspeptin antibody was preadsorbed with RFRP peptide to examine potential cross-reactivity. This procedure did not result in a change in the pattern or intensity of kisspeptin labeling, indicating that the antibody does not exhibit cross-reactivity with this peptide.

Light microscopy

Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm) using a Zeiss Z1 microscope (Thornwood, NY). Every fourth section through the AVPV (AVP/kisspeptin, VIP/kisspeptin, or kisspeptin/FOS) and the medial septum (MS)/diagonal band of Broca (DBB), and medial preoptic area (MPOA; GnRH/FOS) were assessed. The transition from MS/DBB to the MPOA was considered to occur at the beginning of the organum vasculosum of the lamina terminalis, a region clearly defined by GnRH labeling. For light microscopy, kisspeptin cells identified as having AVP or VIP contacts or expressing FOS or GnRH cells expressing FOS were digitally captured at ×400 (fiber contacts) or ×200 (FOS) in 8-bit gray scale using a cooled charge-coupled device camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined by two independent observers using Photoshop software (Adobe Systems, Inc., San Jose, CA) to view CY-2 and CY-3 channels independently. Variation between observers was 1.4% for single-cell analysis and 3.7% for double-labeled analyses. Kisspeptin or GnRH cells with a clear nucleus were quantified using single-channel analysis. Cells were considered to be double labeled if FOS was expressed in the cell nucleus but not beyond the borders of each predefined nuclear area. All kisspeptin cells identified as having putative AVP or VIP contacts were examined using confocal analyses (see below). For FOS expression, only those kisspeptin and GnRH cells with a visible nucleus in which FOS expression was localized to the nucleus were counted as double-labeled cells.

Confocal microscopy

Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss) with a Zeiss LSM 510 laser-scanning confocal attachment. The sections were excited with an argon-krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked images were collected as 0.5 μm (fiber assessment) or 1.0 μm (FOS expression) multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. Kisspeptin or GnRH cells in a given brain region were examined through their entirety. To examine SCN contacts, kisspeptin cells with putative AVP or VIP contacts were scanned though the extent of each cell in 0.5 μm

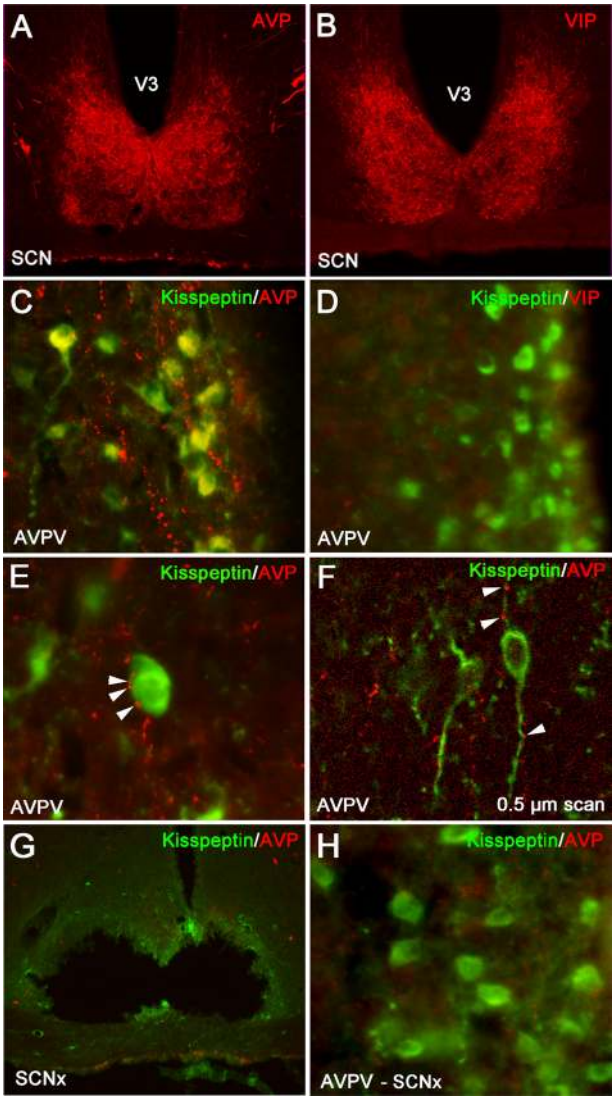

FIG. 2. Kisspeptin-ir cells in the AVPV receive SCN-derived fiber contacts expressing AVP-ir but not VIP-ir. A and C, Low-power photomicrographs of AVP-ir in the SCN (A) and in the AVPV (C), in which kisspeptin cell bodies receive extensive AVP-ir fiber contacts. B and D, Low-power photomicrographs of VIP-ir in the SCN (B) and in the AVPV (D), in which VIP-ir is virtually nonexistent around kisspeptin-ir cell bodies. E, High-power photomicrograph showing several presumptive AVP-ir terminal boutons on a kisspeptin-ir cell body. *Arrows* are indicative of close contacts. F, Confocal image (0.5 μm scan taken at ×400) confirming AVP-ir contacts upon kisspeptin-ir cell body and processes. *Arrows* are indicative of presumptive boutons. G and H, Low-power photomicrographs of AVP-ir in an SCN-lesioned hamster, at the level of the SCN (G), in which AVP-ir is maintained in the paraventricular nucleus and supraoptic nucleus, and in the AVPV (H), in which AVP-ir contacts upon kisspeptin-ir cell bodies is virtually eliminated after SCN lesions, confirming the AVP-ir fibers contacting kisspeptin-ir cell bodies originates from the SCN in C, and see *Results*.

increments at ×400. Only those cells in which the AVP- or VIP-labeled fiber contacted a kisspeptin cell in the same 0.5-μm scan were counted as close contacts. Cells characterized as double labeled for FOS/kisspeptin or FOS/GnRH at the conventional microscopy level were confirmed in the same manner to ensure that FOS was expressed within the cells rather than in overlapping cells in the same light microscopic field of view. Likewise, cells classified as single labeled were assessed to ensure that the conventional microscopy strategy did not result in false-negative results. At least 10% of cells quantified using conventional microscopy were assessed in confocal scans for FOS colabeling. Regions of the brain with putative double label identified at the light level were scanned in 1.0-μm steps at ×400.

Statistics

Data were analyzed using SigmaStat software (Aspire Software, Intl., Ashburn, VA) for all studies. Data for kisspeptin cell counts, FOS expression in kisspeptin cells, and GnRH cells were analyzed using 2 × 4 (hormonal condition × time of day) ANOVA for those studies assessing the change in activation over the course of the day. Total cell counts and FOS expression in GnRH and kisspeptin cells were analyzed using 2 × 2 (treatment × time of day) AVOVA for studies after the injection of AVP. Data for total cell counts and FOS expression in GnRH cells were analyzed using 2 × 2 × 2 ANOVAs (hormonal condition × treatment × time of day) for studies after the injection of kisspeptin. Group differences were evaluated using Tukey honestly significant difference tests. Differences were considered significant if $P < 0.05$.

Results

The daily activational state of AVPV kisspeptin cells is coordinated with the LH surge and is estradiol dependent

Daily fluctuations in kisspeptin neuronal cell activity were assessed at time points before, during, and after the time of the LH surge (ZT 7, 11, 13, 16) in OVX hamsters provided with either empty (Fig. 1, A–D) or estradiol-filled (Fig. 1, E–H) capsules. Syrian hamsters held in a 14-h light, 10-h dark cycle express LH concentrations on proestrous that peak 4 h before onset of darkness, with an initial increase in LH around 6 h before darkness and cessation of the surge approximately 2 h before lights out (13, 50). In hamsters provided with empty capsules and those with capsules containing estradiol, the percentage of kisspeptin cells expressing FOS exhibits a significant daily pattern consistent with a circadian-controlled mechanism participating in LH surge initiation (Fig. 1I). Neuronal activation was low in the early afternoon (ZT 7), increased markedly around the time of the LH surge (ZT 11 and ZT 13), and decreased 2 h after lights out (ZT 16). OVX hamsters exhibited a significantly lower percentage of kisspeptin cells expressing FOS than OVX + estradiol treated females at each time point measured ($P < 0.05$ in each case), with the exception of (ZT 7 (Fig. 1I). The total number of kisspeptin-ir cells was not impacted by time of day or hormonal condition ($P > 0.05$ in each case; Fig. 1J).

Kisspeptin cells in the AVPV are contacted by SCN-derived AVP, but not VIP, fibers

AVPV kisspeptin cells received extensive contacts (37.7 ± 6.3%) from AVP-ir fibers (Fig. 2C). All contacts

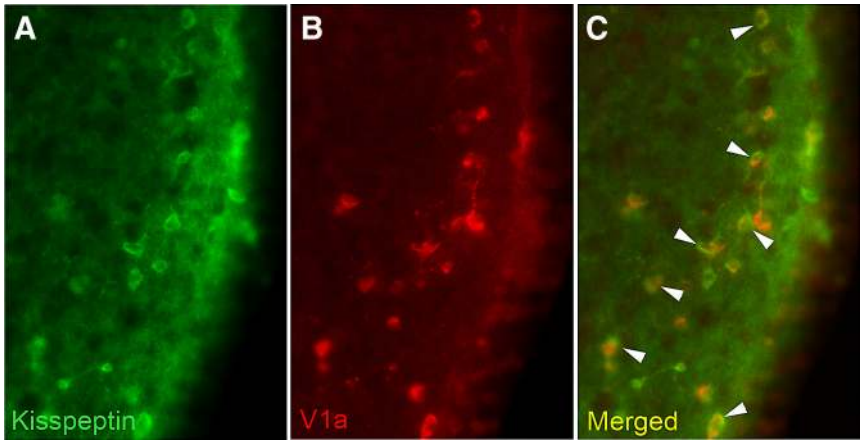

FIG. 3. Kisspeptin cells in the AVPV express the V1a receptor. Low-power photomicrographs of kisspeptin-ir cells in the AVPV (A), V1a-ir cells in the AVPV (B), and the merged image showing overlap between kisspeptin-ir and V1a-ir (C).

at the light level were confirmed to be in the same 0.5 μm plane by confocal microscopy (Fig. 2F). Cells not exhibiting contacts at the light microscopic level did not have contacts at the confocal level. Conversely, in both light and confocal analyses, kisspeptin-ir neurons in the AVPV did not receive any contacts from VIPergic fibers (Fig. 2D), despite prominent VIP-ir fiber staining in relevant brain loci (MPOA, SCN, subparaventricular zone; Fig. 2A). Given that AVP-ir, but not VIP-ir, fibers contact AVPV kisspeptin cells, we sought to determine whether this cell population expresses the main AVP receptor subtype, V1a. We found that 42 ± 7.4% of kisspeptin-ir cells exhibited V1a-ir labeling (Fig. 3). To assess whether AVP-ir contacts on kisspeptin cell bodies in the AVPV originate from the SCN, electrolytic lesions were directed at the SCN, and double-labeled immunocytochemistry was performed for kisspeptin and AVP. Hamsters with lesions sparing the SCN served as controls. AVP-ir fiber appositions on kisspeptin cell bodies were eliminated in SCN-lesioned hamsters (Fig. 2, G and H). Importantly, SCN lesions eliminating AVP-ir contacts on kisspeptin cells spared the supraoptic nucleus and paraventricular nucleus (Fig. 2G).

Central vasopressin administration reveals indiscriminate activation of kisspeptin neurons concomitant with gated activation of the GnRH system

Kisspeptin neuronal activation was assessed following intracerebroventricular injections of saline or AVP at ZT 1 and ZT 11 in SCN-intact, OVX + estradiol females (Fig. 4). At ZT 1, the percentage of kisspeptin-ir cells expressing FOS was significantly increased over saline controls ($P < 0.05$) (Fig. 4B). At ZT 11, kisspeptin cells were maximally activated, with no differences observed between AVP-treated and saline controls ($P > 0.05$; Fig. 4B). In contrast, AVP infusions did not increase GnRH cell activation at ZT 1 relative to saline controls (Fig. 4B) ($P > 0.05$). At ZT 11, both GnRH and kisspeptin cells were maximally activated, with no differences between saline or AVP-treated females ($P > 0.05$ in both cases) (Fig. 4B). Total GnRH-ir and kisspeptin-ir cell counts did not differ significantly across treatments or time periods ($P > 0.05$ in all cases) (Fig. 4, C, and D), indicating differences in FOS-ir are representative of the proportional changes in activation states in all cases.

The GnRH system exhibits time-dependent sensitivity to kisspeptin stimulation

To examine whether the GnRH system is differentially sensitive to kisspeptin treatment at times when the LH surge cannot (ZT 1) or can (ZT 11) be induced, GnRH neuronal activation was assessed after injections of saline or kisspeptin (Fig. 5 and Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The estrogen dependence of any differences was examined in OVX females treated with an empty or estradiol-filled capsule. In all cases, excluding the 4nmol dose of kisspeptin in the MS/DBB of OVX hamsters and the preoptic area (POA) of OVX + estradiol hamsters, kisspeptin was more effective at activating the GnRH system at ZT 11 than at ZT 1 ($P < 0.05$ in each case; Fig. 5, A and B). In both brain loci and both time points, estrogen increased the percentage of GnRH neurons expressing FOS in vehicle-treated controls and 2 nmol kisspeptin-treated hamsters compared with OVX females bearing empty capsules ($P < 0.05$ in all cases). The presence of estrogen also increased the percentage of GnRH neurons expressing FOS at the 4-nmol dose of kisspeptin relative to OVX females ($P < 0.05$). In OVX + estradiol hamsters probed at ZT 11, there were no differences among groups in either brain region, suggesting that the GnRH systems is maximally activated at this time due to endogenous stimulation of the GnRH in the presence of estradiol ($P > 0.05$ in all cases). Finally, the total number of GnRH cells was not affected by time, kisspeptin treatment, or estradiol ($P > 0.05$ in all cases) (Fig. 5, C and D).

Discussion

The present findings reveal a hierarchical organization of the hamster ovulatory circuitry involving interactions among the circadian system, a sex steroid integration center, and a final common pathway gating information flow.

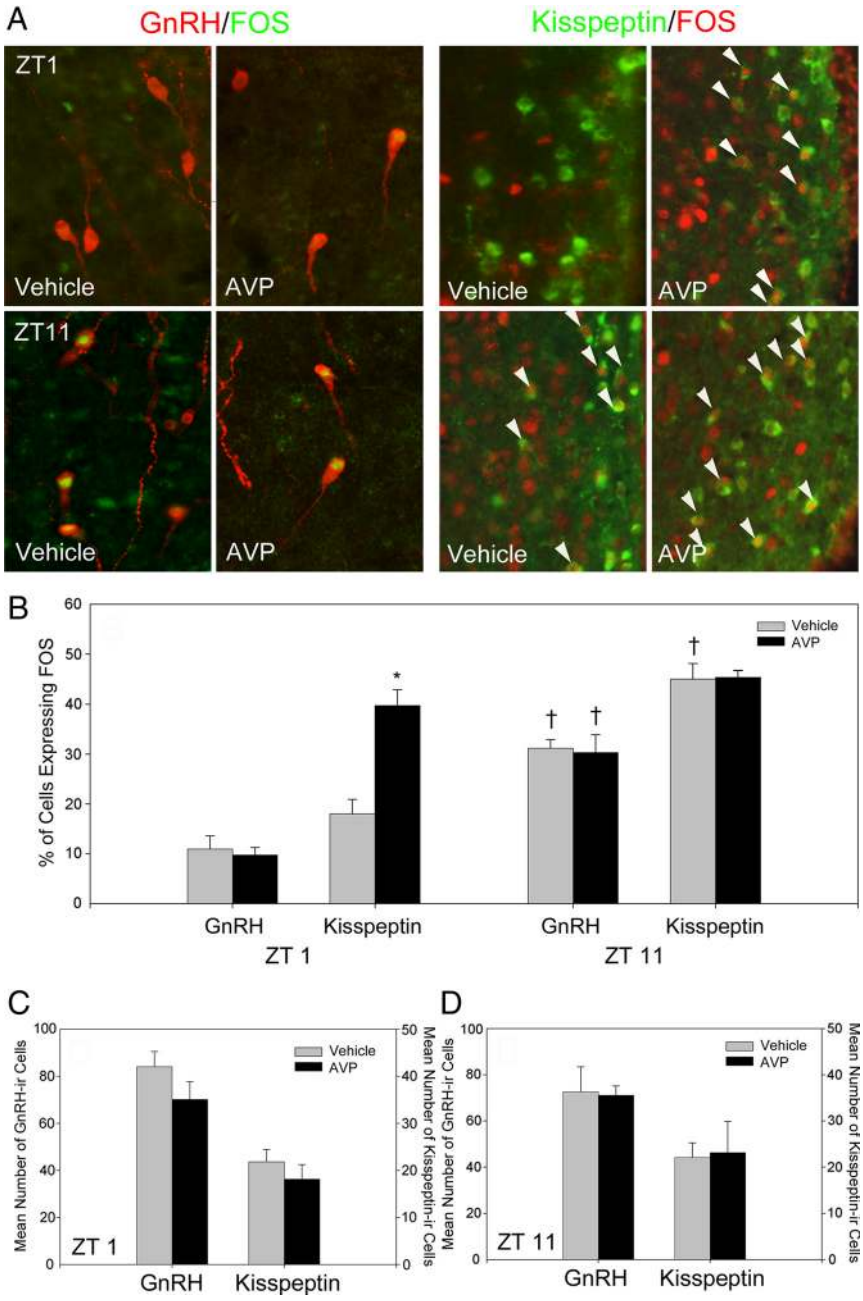

FIG. 4. Central vasopressin administration reveals indiscriminate activation of kisspeptin neurons concomitant with gated activation of the GnRH system. Intracerebroventricular administration of AVP robustly activates kisspeptin-ir cells in the morning (ZT 1), whereas GnRH-ir cells remain inactive at this time point. In the afternoon (ZT 11), both kisspeptin-ir and GnRH-ir cells express high levels of FOS activation after saline or AVP injection. A (*left panel*), Low-power photomicrographs of GnRH-ir cells expressing FOS after i.c.v. saline (vehicle) or AVP administration at ZT 1 or ZT 11. A (*right panel*), Low-power photomicrographs of kisspeptin-ir cells in the AVPV expressing FOS after i.c.v. saline (vehicle) or AVP administration at ZT 1 or ZT 11. (B) Mean (±SEM) percentage of GnRH-ir and kisspeptin-ir cells expressing FOS after saline or AVP at ZT 1 or ZT 11. C and D, Mean (±SEM) total number of GnRH-ir and kisspeptin-ir cells after saline or AVP at ZT 1 (C) or ZT 11 (D). *, Significantly greater than the percentage of kisspeptin cells expressing FOS in hamsters treated with vehicle at ZT 1 ($P < 0.05$); †, significantly greater than females given the same pharmacological treatment at ZT 1 ($P < 0.05$).

First, we find that AVPergic cells originating in the SCN target kisspeptin cells in the AVPV, a subpopulation that express the vasopressin receptor subtype, V1a. The AVPV population of kisspeptin neurons exhibits a daily pattern of activation with peak activity coincident with the timing of the GnRH/LH surge (13). The daily pattern of kisspeptin cellular activity is partially dependent on the presence of estradiol; ovariectomy markedly attenuates, but does not abolish, this daily activation. We also found that GnRH neurons act as gatekeepers, restricting the preovulatory LH surge to the late afternoon, at least in part, through daily changes in responsiveness to kisspeptin. More specifically, central administration of AVP increases kisspeptin cellular activation at both time points examined, whereas GnRH neurons are sensitive to AVP administration only during the afternoon, suggesting insensitivity to AVP-induced kisspeptin at this time. Finally, pharmacological studies of OVX and OVX/estrogen-primed hamsters confirm daily changes in GnRH cell responsiveness to kisspeptin that are dependent on the presence of estradiol. Together these findings point to kisspeptin neurons in the AVPV as an integration center for estradiol and circadian signals necessary for the generation of the LH surge and suggest that daily changes in GnRH neuron sensitivity to kisspeptin signaling further ensures appropriate timing of the surge (Fig. 6).

In spontaneously ovulating rodents (*e.g.* rats, mice, hamsters), the SCN initiates the LH surge on the day of proestrus when estrogen concentrations are elevated (1). That estrogen is permissive for circadian stimulation of the GnRH system is somewhat paradoxical because estrogen negatively regulates GnRH secretion throughout the remainder of the cycle (51). We have previously shown that this feat is accomplished, at least in part, by the removal of inhibitory influences of the mammalian ortholog of avian gonadotropin inhibitory hormone, RFRP-3, at the time of the surge (13). Historically it was believed that direct VIPergic SCN projections targeting GnRH neurons were responsible for the positive arm of the ovulatory circuit (21, 52). However, given the paucity of ER

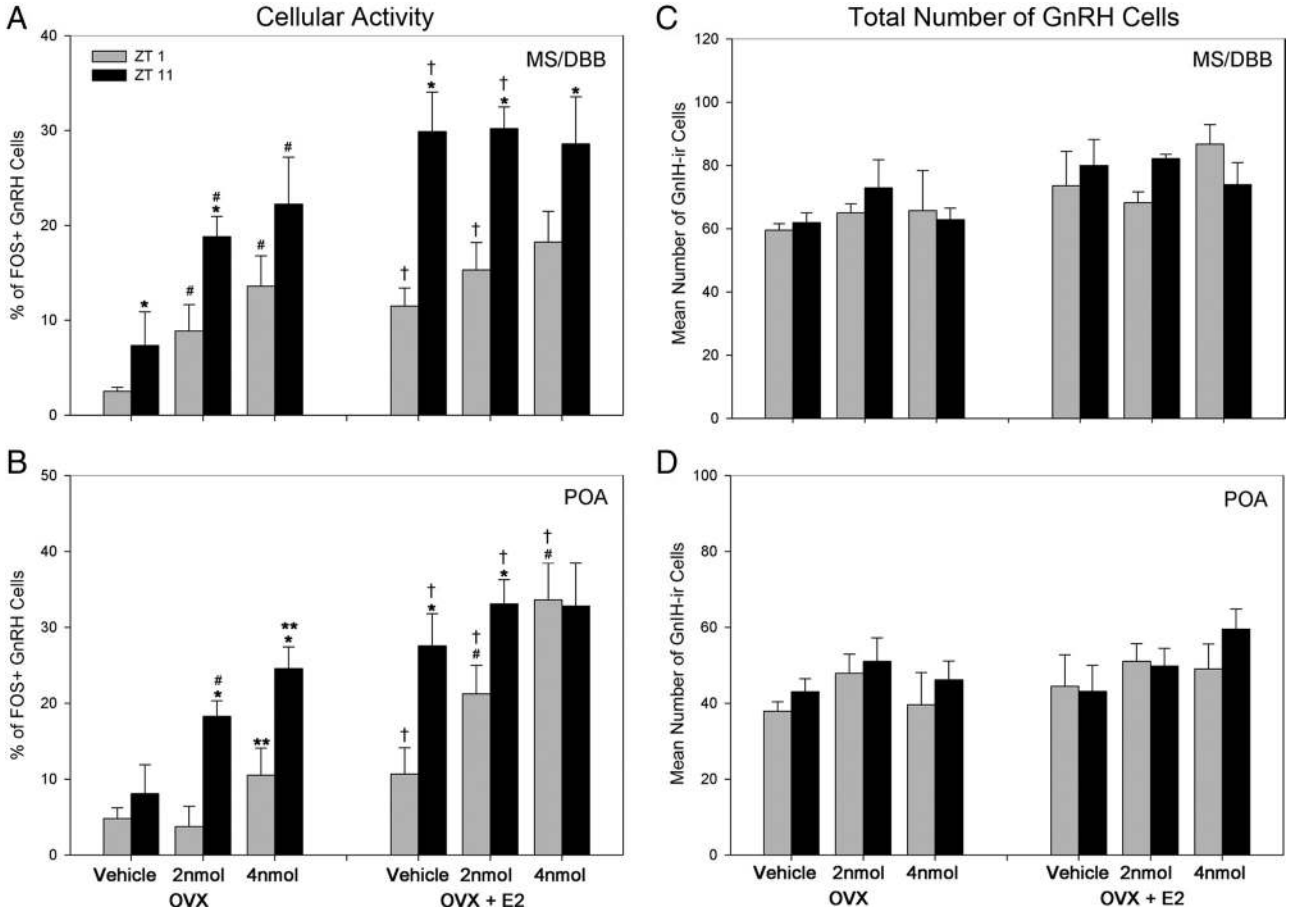

FIG. 5. The activation of GnRH after kisspeptin administration is time dependent, shows regional differences, and is enhanced by the presence of estradiol. A and B, The percentage of GnRH-ir cells expressing FOS after vehicle, 2 nmol, or 4 nmol kisspeptin administration at ZT 1 or ZT 11 in OVX and OVX+ estradiol (E2) hamsters. A, Mean (±SEM) percentage of MS/DBB GnRH-ir cells expressing FOS after kisspeptin administration at ZT 1 or ZT 11 in OVX (*left panel*) and OVX+E2 (*right panel*) hamsters. B, Mean (±SEM) percentage of POA GnRH-ir cells expressing FOS after kisspeptin administration at ZT 1 or ZT 11 in OVX (*left panel*) and OVX+E2 (*right panel*) hamsters. GnRH activation after kisspeptin administration is time dependent in OVX hamsters and is time dependent after 2 nmol kisspeptin in OVX+E2 hamsters. C, Mean (±SEM) total GnRH-ir cells in the MS/DBB after kisspeptin administration. D, Mean (±SEM) total GnRH-ir cells in the POA after kisspeptin administration. No differences were seen between cell counts, indicating the differences in FOS-ir represent proportional changes in GnRH activation levels. *, Significantly greater than hamsters provided with the same pharmacological treatment, at the same time point, and the same hormonal condition ($P < 0.05$); #, significantly greater than vehicle controls at the same time point and hormonal condition; **, significantly greater than vehicle controls and 2 nmol kisspeptin-treated hamsters at the same time point and same hormonal condition; †, significantly greater than OVX hamsters at the same time point and pharmacological treatment. All differences are significant at $P < 0.05$.

expression in GnRH cells (25, 26), researchers began searching for estrogen-responsive targets of the SCN upstream of the GnRH system. Kisspeptin cells in the AVPV represented a likely candidate because this brain tissue expresses FOS coincident with the surge (29), contains ERs (53), and receives SCN input (30, 31). Likewise, kisspeptin cells are up-regulated by estrogen and express FOS at the time of the LH surge (36, 41), suggesting an important role in LH surge initiation. Whether AVPV kisspeptin cells represent an integration point for circadian/sex steroid signaling and might participate in the gating of circadian communication has not been explored.

Because AVPergic cells in the SCN project to the AVPV across species (32, 33) and AVP can only induce the LH surge during a limited time window in estradiol-implanted rats (43), we asked whether this population of kisspeptin cells might receive AVPergic SCN input and gate responsiveness to this peptide. We find that kisspeptin cells receive AVPergic SCN input and express V1a receptors, providing a direct means of communication from the circadian clock to this cell population. Recent findings in mice indicate that SCN-derived AVP cells project to the kisspeptin system (54), suggesting a common mechanism of control across rodent species. We also found that intracerebroventricular (i.c.v.) injections of AVP increase kisspeptin cellular activity. Importantly, although AVP can initiate the LH surge only during the afternoon in estrogen-treated animals (43), administration of this peptide enhances kisspeptin cellular activity in the morning (Fig. 4). In contrast, although kisspeptin activity was increased by AVP administration in the morning, GnRH

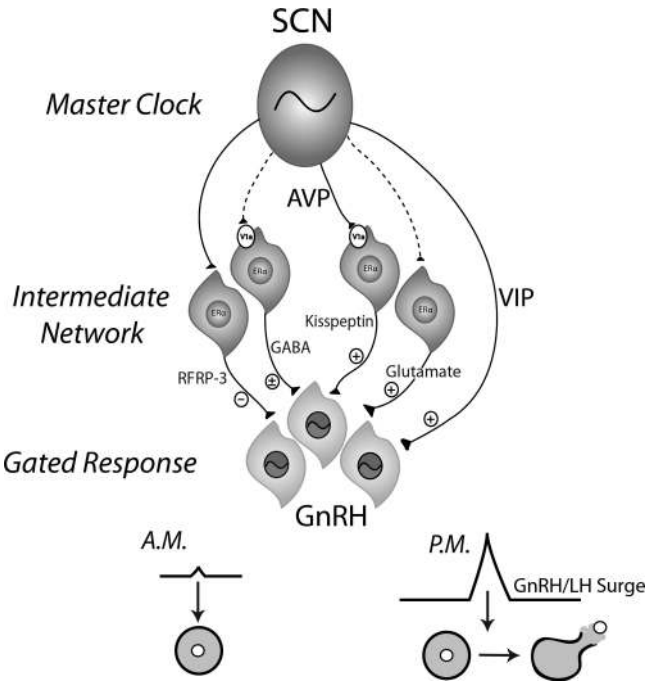

FIG. 6. Model for the circadian control of the GnRH/LH surge. Proposed model by which several well-characterized, circadian-controlled neurochemicals participate in ovulation. The present studies and previous work indicate that the SCN sends efferent projections to kisspeptin neurons in the AVPV that express the V1a receptor and RFRP-3 (*i.e.* gonadotropin inhibitory hormone) neurons in the dorsomedial hypothalamus. GABAergic and glutamatergic neurons within the AVPV likely receive SCN input, but these pathways have not been explored (indicated by *dashed lines*). Together these intermediate signals represent integration sites of circadian and estrogenic input, with all neuronal phenotypes expressing estrogen receptors. GnRH neurons, in turn, receive input from these inhibitory and stimulatory afferents as well as direct VIPergic input from the SCN. In turn, the consequent response of the GnRH system will depend on the sum total of positive and negative influence and on time of day, the latter due to an inherent daily timing mechanism in GnRH cells.

activity was not. As expected, given endogenous AVPergic signaling during the afternoon, both GnRH and kisspeptin activity are maximal in both vehicle- and AVP-treated hamsters. This finding suggested that circadian information is not gated at kisspeptin cells but downstream of this cell population. Because GnRH neurons are the major target of kisspeptin (47, 48), this cell population represented a possible gating locus mediating the timing of ovulation.

Using immortalized GT1–7 cells, we documented that GnRH neurons exhibit time-dependent sensitivity to upstream stimulatory signals that initiate the LH surge, including kisspeptin and VIP (55). GnRH cells express the same clock genes driving circadian function at the cellular level (55–57), providing a potential time-keeping mechanism necessary to appropriately phase daily changes in sensitivity to upstream signaling. By administering kisspeptin in both the morning and afternoon to OVX hamsters with and without concurrent estrogen treatment, several findings emerged that enhance our understanding of the mechanisms that time ovulation (Fig. 5). In the morning (ZT 1), GnRH neurons are considerably less responsive to kisspeptin administration than the afternoon (ZT 11), during which treatment reliably enhances GnRH cellular activity. This attenuated responsiveness is unlikely due to ineffective doses of kisspeptin because previous studies indicated that the GnRH system responds robustly to doses as low as 1 nmol in rats and 1 fmol in mice (36, 58). The presence of estradiol generally enhanced GnRH cellular activity in kisspeptin and vehicle-treated hamsters. These findings suggest that the gating of GnRH activation may occur via circadian-mediated differences in kisspeptin receptor expression or high inhibitory tone during the morning. Notably, a reliable dose-response to kisspeptin is observed in the POA population of GnRH neurons of vehicle-treated hamsters at hamsters at ZT 11 but not ZT1 (Fig. 5B). As expected, during the afternoon when endogenous release of AVP and kisspeptin are maximal, estrogen serves a permissive role in GnRH cell activation, leading to elevated GnRH activity, regardless of treatment (Fig. 5B), thereby masking a dose response. Importantly, that OVX hamsters (Fig. 5B, *left panel*) exhibit a dose response to kisspeptin at ZT 11 further suggests that the endogenous activation of GnRH in estrogen-implanted hamsters accounts for the ceiling effect in estradiol-implanted animals. Whether GnRH gates responsiveness to all upstream signaling represents an important area for further inquiry.

If the LH surge is initiated solely by the integration of estradiol with circadian signaling at AVPV kisspeptin neurons, all downstream effects should reflect the extent of kisspeptin release, and kisspeptin treatment should maximize GnRH cellular activity at any time of day, even in the absence of estrogen. The fact that the addition of estradiol potentiates kisspeptin-stimulated GnRH cellular activity suggests additional, estrogen-dependent mechanisms of stimulatory control. ERs have been localized to the SCN of humans (59), mice (60), and rats (61), suggesting that AVP or VIP neurons might be directly responsive to this sex steroid. Likewise, in addition to the requirement of estradiol to maximize GnRH cellular activity, the GnRH response to kisspeptin stimulation is time dependent, suggesting that the surge is not simply elicited when the GnRH system is stimulated with kisspeptin but that the GnRH system gates daily responsiveness to this peptide. These findings agree with our previous findings of daily changes in the sensitivity of the GnRH system to kisspeptin administration using GT1–7 cells (55), and studies in which central infusion of kisspeptin i.c.v. (62) or into the mPOA (63) fail to advance the onset of the LH surge in either

naturally cycling or OVX and estradiol/progesterone-primed female rats.

Recent findings in female mice provide converging support for the present results implicating circadian control of kisspeptin in ovulation. *Kiss1/c-fos* mRNA is maximally coexpressed to approximately 40% around the time of the GnRH surge when mice are held in constant conditions (64), indicating circadian control of kisspeptin cellular activity. Our immunohistochemical studies indicate a maximum coexpression of kisspeptin/FOS of 32% around the time of the LH surge, slightly lower than the maximal *Kiss1/c*-fos mRNA coexpression previously reported. The disparity between these findings and the present findings may be the result of posttranscriptional *Kiss1* regulation, leading to a lower number of cells expressing the mature peptide. Additionally, it is possible our quantification strategy was more conservative, restricting our quantification to only those FOS-labeled cells with a clear nucleus. In this same report, ovariectomy without estradiol replacement abolished this daily pattern. The latter finding is at odds with the present result, indicating that the rhythm of kisspeptin/FOS is grossly attenuated, but maintained, in OVX hamsters not treated with estradiol. This partial dependence on estrogen is consistent with the daily pattern of LH secretion that persists, albeit with a lower amplitude than estradiol-implanted animals, in OVX Syrian hamsters (50). Whether this disparity is due to species differences or a discrepancy in transcriptional/translational differences in the daily pattern of *Kiss1*/kisspeptin represents an interesting question for further exploration.

It is likely that other neural loci upstream of the GnRH systems are targets of the SCN involved in the timing of the LH surge. Dual-phenotype neurons expressing γ-aminobutyric acid (GABA)/glutamate within the AVPV, for example, have been implicated in the control of GnRH activity and are regulated by estradiol feedback (65–67). Likewise, GnRH neurons respond to GABA differentially across the day, with excitatory responses in the afternoon (51, 68). Although no direct connections have been reported between the SCN and GABA neurons within the AVPV, the diurnal shift in GABA release (68) and the expression of V1a receptors in GABA neurons within the AVPV (69) point to a potential circadian mechanism regulating this cell population. Furthermore, kisspeptin up-regulates GABA transmission in the AVPV during estrogen-negative (but not positive) feedback, further suggesting a local control within the AVPV (70). In apparent contrast to the present findings, in one previous study, unilateral AVPV administration of a V1aR antagonist did not alter the timing or amplitude of the LH surge in rats (43). However, because the V1aR antagonist was injected unilaterally and the position of the dialysis probe was variable, it is unclear to what extent the full population of kisspeptin cells was impacted. Other studies of rats indicate that bilateral suppression of V1aR attenuates the LH surge in proestrous rats (71). Additionally, in cocultures of POA and SCN, the GnRH surge is coordinated with the rhythm in AVP, but not VIP (72), providing further evidence for an important role of AVP in surge generation.

The present studies reveal a novel, circadian-controlled neurochemical pathway participating in the LH surge. Kisspeptin neurons in the AVPV are targets of AVPergic SCN fibers and express V1a receptors, providing evidence for a direct connection between the master circadian clock and kisspeptin neurons in the AVPV. Kisspeptin neurons exhibit a daily activation pattern coincident with the timing of the GnRH/LH surge that is dependent on the presence of estradiol, further implicating these neurons as integrators of circadian and estrogenic signals necessary for the preovulatory LH surge. AVPV kisspeptin cells are indiscriminately activated by SCN peptidergic signaling, whereas the GnRH system displays time-dependent responsiveness to AVP and kisspeptin stimulation. Together these findings point to a novel mechanisms of ovulatory control whereby circadian and estrogenic signals converge on AVPV kisspeptin cells and kisspeptin signaling is gated by daily changes in GnRH cell sensitivity to this peptide.

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