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The Role of Kisspeptin and RFRP-3 Neurons in the Circadian-Timed Preovulatory Luteinizing Hormone Surge

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Abstract

Many aspects of female reproduction often require intricate timing, ranging from temporal regulation of reproductive hormone secretion to the precise timing of sexual behavior. In particular, in rodents and other species, ovulation is triggered by a surge in pituitary luteinizing hormone (LH) secretion that is governed by a complex interaction between circadian signals arising in the hypothalamus and ovarian-derived estradiol signals acting on multiple brain circuitries. These circadian and hormonal pathways converge to stimulate a precisely-timed surge in gonadotropin-releasing hormone (GnRH) release (i.e., positive feedback), thereby triggering the preovulatory LH surge. Reflecting its control by afferent circadian signals, the preovulatory LH surge occurs at a specific time of day, typically late afternoon in nocturnal rodents. Although the specific mechanisms mediating the hormonal and circadian regulation of GnRH/LH release have remained poorly understood, recent findings now suggest that estradiol and circadian signals govern specific reproductive neuropeptide circuits in the hypothalamus, including the newly-identified kisspeptin and RFamide-related peptide-3 (RFRP-3) neuronal populations. Neurons producing kisspeptin, the protein product of the *Kiss1* gene, and RFRP-3 have been shown to provide excitatory and inhibitory input to GnRH neurons, respectively, and are also influenced by sex steroid and circadian signals. Here, we integrate classic and recent findings to form a new working model for the neuroendocrine regulation of the circadian-timed preovulatory LH surge in rodents. This model proposes kisspeptin and RFRP-3 neuronal populations as key nodal points for integrating and transducing circadian and hormonal signals to the reproductive axis, thereby governing the precisely-timed LH surge.

Keywords

kisspeptin; *Kiss1*; GnIH; RFRP-3; GnRH; SCN; clock; ovulation

Introduction

Gonadotropin hormones secreted by the pituitary govern gonadal physiology, including ovulation. Gonadotropins themselves are regulated by forebrain neurons that secrete gonadotropin-releasing hormone (GnRH). In rodents, at most times during the estrous cycle, low levels of estradiol (E_2) secreted from the ovaries provide negative feedback on the upstream brain circuits that regulate GnRH release, thereby regulating tonic pulsatile GnRH

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secretion. However, in females during proestrus, rising levels of E_2 paradoxically provide positive feedback signals to the GnRH system, resulting in substantially increased GnRH secretion (1-3). This positive feedback-induced GnRH surge in turn causes the pituitary to generate a surge in luteinizing hormone (LH) secretion, which induces ovulation (4, 5).

As with most physiological processes, reproduction—and ovulation in particular—requires exquisite timing and regulation. In female rodents, ovulation is timed to occur in the hours just prior to or coincident with female sexual behavior, which occurs primarily on late proestrus and early estrus, when E_2 levels are elevated. This coordinated timing of ovulation and subsequent sexual behavior ensures that the two processes are temporally synchronized with each other, thereby enhancing the probability of fertilization after mating (6). The preovulatory LH surge is therefore optimally timed; in nocturnal rodents, it reliably occurs in the late afternoon or early evening of proestrus, just prior to mating. Ovariectomized (OVX) female rodents completely lacking E_2 do not exhibit an LH surge, highlighting the essential requirement for elevated E_2 in the generation of the LH surge (7-9). However, similar to gonadally-intact proestrus females, OVX female rodents that are supplemented with elevated levels of E_2 (OVX+E_2) display robust LH surges that occur once daily at a specific time, exclusively around the onset of darkness (7-9). This precisely-timed nature of preovulatory LH release suggests involvement of the circadian system, specifically the suprachiasmatic nucleus (SCN), the circadian pacemaker located in the mammalian hypothalamus. Here we evaluate the current knowledge of the neuroanatomical, cellular, and molecular mechanisms underlying the generation and timing of the preovulatory LH surge in rodents, focusing on recently-identified signaling factors and circuits in the hypothalamus.

Circadian Component of the LH Surge

Brief overview and functional anatomy of the SCN, the mammalian circadian clock

In mammals, the circadian pacemaker located in the SCN of the hypothalamus governs the circadian rhythms of most biological processes, from gene expression to overt behavior. Several lines of evidence accumulated over the past four decades, primarily from rodent models, demonstrate the importance of the SCN as a central circadian pacemaker. First, in both light-dark cycles and static environmental conditions (such as constant darkness), the SCN demonstrates near-24 h oscillations in neuronal activity, glucose consumption, and gene expression (10-16). Second, *in vivo* ablation or surgical isolation of the SCN leads to arrhythmia in brain electrical activity, physiology, and overt behavior (17-20). Third, transplanting fetal SCN tissue into an arrhythmic host animal with an ablated SCN restores circadian rhythmicity in the host animal with the donor animal's period (21-24). Fourth, SCN tissue explants retain near 24-h rhythmicity in electrical activity and neuropeptide release *in vitro*, demonstrating that SCN cells are themselves autonomous clocks that do not rely on external neural or hormonal input to generate rhythmicity (25-27). Lastly, the external environment—and in particular, light—can modulate and synchronize the period and phase of circadian rhythms, and supporting its role as the circadian clock, the SCN receives direct light input from melanopsin-containing photosensitive cells in the retina (via the retinohypothalamic tract) (28, 29).

The SCN is functionally divided into two parts: the ventrolateral "core" and the dorsomedial "shell." The core neurons receive direct light input from the retinohypothalamic tract, and then transmit this information to the shell neurons via extensive neural projections (30). Although less common, some neural connections also project back from the shell to the core (31). Intriguingly, in both the core and the shell, each SCN neuron is rhythmic on its own (32); however, by synchronizing themselves as an interactive network, SCN neurons produce rhythmic output that is precise, robust, and more immune to disruptions (33). Neural fibers from cells in the SCN project to numerous brain areas, including other

hypothalamic regions, such as (but not limited to) the subparaventricular zone, paraventricular nucleus (PVN), preoptic area, the periventricular nucleus, and the dorsomedial nucleus (DMN) (30, 34).

Communication of temporal information within and from the SCN relies on the activity of different neurotransmitters and neuropeptides within the SCN. The SCN produces several neuropeptides such as gastrin-releasing peptide, substance P, and prokineticin 2, as well as GABA (35, 36). However, two other SCN neuropeptides, vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP), have been most strongly implicated in circadian rhythm biology. These two neuropeptides are synthesized in different regions of the SCN, with core and shell SCN neurons expressing and releasing VIP and AVP, respectively (37). In rodents, the SCN as a whole highly expresses $VPAC_2R$, a VIP receptor subtype (38, 39), and both VIP and $VPAC_2R$ mRNA expression levels, as well as VIP release, demonstrate *in vitro* circadian rhythmicity in SCN slices (40-42). Within the SCN, VIP neurons receive and communicate photic information from the core to shell neurons, as well as to other core neurons, and may also communicate circadian information to downstream tissues, including reproductive circuits (see below) (43). Indeed, evidence from transgenic mice lacking the gene for either VIP or $VPAC_2R$ implicates VIP as an important communicator to extra-SCN areas because these mice display disrupted or altered circadian rhythms, and sometimes arrhythmicity (44, 45).

AVP, highly expressed in the SCN shell, demonstrates circadian rhythmicity *in vivo*, with highest levels during the subjective day (46). However, AVP is unlikely to contribute to endogenous rhythm generation within the SCN since the AVP-deficient Brattleboro rat displays intact circadian rhythmicity for several behaviors (47, 48). Yet, AVP may enhance the SCN's firing rate since several studies demonstrate an excitatory response of SCN neurons to AVP (49-51). Moreover, AVP serves as an important output signal of the SCN and has been implicated in regulating several circadian rhythms, including circadian stress responses (52-54) and perhaps reproductive function (discussed below). Supporting this role, SCN neurons containing AVP project to several hypothalamic areas, including the PVN, subPVN, preoptic area, AVPV, and the DMN (55, 56).

Relation between the circadian clock and the LH surge

There is good evidence that the circadian clock in the SCN governs the timing of the preovulatory LH surge in rodents. First, a series of groundbreaking experiments performed over half a century ago in female rodents demonstrated that not only does the LH surge reliably occur in the late afternoon of proestrus, but treatment with dibenamine, atropine, or barbiturate delays the LH surge approximately 24 h until the late afternoon of the next day (57-61), suggesting a circadian regulation of the events generating the LH surge. Second, OVX rodents treated with constant levels of elevated E_2 demonstrate a reliable late afternoon LH surge which repeats daily at the same exact time, also hinting at an underlying circadian component (9, 62, 63). Third, SCN neurons are activated immediately before the LH surge, as demonstrated by increased c-Fos expression (a marker of general cell activation) (64). Fourth, experimentally-induced phase shifts in the circadian rhythmicity of behaviors known to be driven by the SCN result in congruent shifts in the timing of the LH surge. For example, in nocturnal rodents, locomotor activity is timed by the SCN and, like the LH surge, begins around the onset of darkness (19, 65-67). Light cycle manipulations that change the period or phase of the circadian locomotor rhythm also modify the timing of the LH surge; however, the new onset of the LH surge is still tightly coupled to the new onset of locomotor activity (68-70). For example, in Syrian hamsters with the circadian τ mutation (which leads to a much shortened locomotor activity rhythm), the onset of the LH surge is still perfectly synchronized with earlier onset of locomotor activity (71). Because the timing of the LH surge is tightly tethered to the onset of locomotor activity, which itself

is known to be timed by the SCN, it is likely that the LH surge is also timed by the SCN. Lastly, physical ablation of the SCN in female Syrian hamsters and rats (72-74), as well as genetic disabling of circadian clock genes in mice (75), abolishes the LH surge and ovulation, even in the presence of E_2 supplementation, suggesting that the SCN clockwork is critical for the occurrence of the LH surge in rodents.

Connectivity of the SCN to GnRH neurons

If, as the evidence above suggests, a circadian pacemaker in the SCN times the LH surge, then how does the SCN communicate with the reproductive axis? Several anatomical connections have been identified from the SCN (both core and shell) to areas potentially involved in generating the LH surge (76-80). At least two physical pathways (direct and indirect) link the SCN to GnRH neurons. The direct pathway includes VIPergic core neurons directly targeting GnRH neurons that express the VIP receptor $VPAC_2R$ (79, 81). There is some evidence, albeit limited, that this VIP connection plays a role in the preovulatory LH surge. First, VIP-targeted GnRH neurons express c-Fos at the time of the preovulatory LH surge, and blocking VIP signaling via *in vivo* antisense antagonism in E_2-treated rats delays the surge (82-84). Second, in line with E_2's requisite role in positive feedback, stimulatory effects of VIP on GnRH neurons are E_2-dependent, evidenced by the finding that VIP treatment promotes electrical activity of GnRH neurons in brain slices of OVX mice only when E_2 is present (85). Third, GnRH neuron electrical responses to exogenous VIP are circadian-timed, with peak responses around the predicted onset of the LH surge (85). Lastly, *vip*-null mice present some degree of infertility (45, 86); however, mice lacking a functional gene for $VPAC_2R$ (*vipr2*-null) show no impairment in fertility (44). On the whole, these data suggest that VIP may facilitate some aspects of the circadian release of GnRH that leads to the preovulatory LH surge, though additional evidence supporting this conjecture is needed.

The indirect pathway linking the SCN and GnRH axis begins with AVPergic shell neurons innervating the hypothalamic anteroventral periventricular nucleus (AVPV), which then projects to GnRH neurons (76-78, 80, 87). Several pieces of evidence strongly suggest that AVP may convey circadian information to the AVPV as part of the LH surge mechanism. First, the AVPV expresses V1a (an AVP receptor subtype), and this V1a expression increases with E_2 treatment in rats and mice (88, 89). Second, the approximate time of peak AVP expression in SCN shell neurons in mice occurs in the late afternoon/early evening, similar to when the LH surge occurs (90). Third, experimentally increasing extracellular AVP levels in the AVPV area with reverse microdialysis produces an LH surge in SCN-lesioned $OVX+E_2$ rats that do not normally display LH surges (91). Fourth, in mice with a mutation in the core circadian gene *Clock* that leads to a lack of preovulatory LH surges, central administration of AVP stimulates LH secretion when given during the afternoon (92). Conversely, central injection of a V1a antagonist in proestrus rats prevents the late afternoon LH surge (93). Although no genetic mouse studies have directly tested the requirement of AVP signaling for the LH surge, homozygous female Brattleboro rats (which have a spontaneous mutation in the AVP gene) have several reproductive impairments, including abnormal estrous cycles and reduced conception rates (94).

The above findings implicate an interconnected neuronal network that guides the circadian regulation of the preovulatory LH surge, with VIP and AVP neurons providing direct and indirect circadian input to GnRH neurons, respectively. However, this picture is complicated by conflicting findings on the exact nature and timing of the actions of VIP and AVP. For instance, while VIP may have an activational effect on GnRH neurons, other evidence suggests that VIP may be inhibitory during the LH surge (84, 95). Likewise, AVP treatment is effective at inducing the LH surge in the late afternoon, but not at other times of the day (96). In addition, the SCN sends axonal fibers to other targets regions besides GnRH

neurons and the AVPV, opening the possibility that additional indirect pathways may be involved in gating the timing of GnRH secretion.

Potential Neural Populations Mediating Circadian Control of GnRH

The kisspeptin system: function and neuroanatomy

A key neuropeptide evidenced to regulate GnRH neurons is the recently-identified kisspeptin. Humans and mice lacking either the kisspeptin receptor, Kiss1R (formerly termed GPR54), or *Kiss1*, the gene that encodes kisspeptin, demonstrate hypogonadotropic hypogonadism, a condition where pubertal maturation and reproductive function are severely impaired due to a deficiency in GnRH secretion (97-100). Kiss1R is expressed in GnRH neurons, indicating the potential for kisspeptin to directly signal to these cells (101, 102). Numerous studies in rodents and other species have shown that kisspeptin directly stimulates GnRH neurons, leading to enhanced GnRH secretion and subsequent gonadotropin secretion (101-106). Likewise, kisspeptin treatment potently activates electrical firing of GnRH neurons in hypothalamic slices (107, 108). These findings indicate that kisspeptin might play a role in generating the LH surge via activation of GnRH neurons. Indeed, blocking kisspeptin signaling with Kiss1R antagonists or kisspeptin antibody treatments alleviates, or in many cases, abolishes the LH surge (109-111). Moreover, *Kiss1R*- and *Kiss1*-null mice do not exhibit LH surges, even after appropriate E_2 treatment (112), suggesting that kisspeptin signaling is indeed a critical component of the LH surge mechanism. However, it should be noted that at least one study is at odds with these findings. Dungan *et al.* reported that OVX+E_2 *Kiss1R*-null mice are surprisingly able to display small LH surges (113). The reasons for these discrepant findings are not known, but may relate to differences in the specific mouse transgenic model or surge paradigm used, both of which were slightly different between the two studies (112, 113).

In rodents, *Kiss1* is expressed in two discrete regions of the hypothalamus, the anatomical continuum comprising the AVPV and the rostral periventricular nucleus (PeN) and, more caudally, the arcuate nucleus (ARC) (103, 114-116), as well as some extra-hypothalamic sites such as the amygdala (117). The source of kisspeptin signaling necessary for specifically promoting the LH surge is likely within the AVPV/PeN, as this region both communicates with GnRH neurons and is critical for generating the LH surge (87). Unlike GnRH neurons, which lack estrogen receptor α (ERα) (118, 119), the ER subtype known to mediate positive feedback, the AVPV/PeN does contain ERα-expressing neurons (120, 121). While the other main ER subtype, ERβ, is also present in the AVPV, antagonism or genetic knockout of this receptor does not prevent LH surges, indicating that ERβ is not critical for the LH surge process (122, 123). Functionally, lesioning the AVPV leads to acyclicity and a lack of E_2-induced LH surges (74, 124, 125). Discrete implantation of E_2 in or near the AVPV induces an LH surge, while microimplants of antiserum against E_2 in the same area inhibits the surge (126-129). The AVPV region as a whole also expresses increased c-Fos at the time of the LH surge, supporting a role for the AVPV in the E_2–mediated positive feedback process (130). Importantly, SCN neurons exhibit increased c-Fos expression just prior to the LH surge, and this expression precedes the subsequent peaks in c-Fos expression in the AVPV and GnRH neurons (64), illustrating the sequential link of activation between the SCN, AVPV, and GnRH neurons.

Emerging evidence now suggests that the c-Fos- and ERα-expressing neurons in the AVPV that participate in the LH surge are kisspeptin neurons. First, *Kiss1*-expressing neurons located in the AVPV/PeN express ERα, and *Kiss1* gene expression in the AVPV/PeN of female rodents increases in response to E_2 (115, 116, 131, 132). Second, *Kiss1* neurons in the AVPV/PeN display c-Fos at the time of the LH surge in proestrous or OVX+E_2-treated females but not in diestrus females or OVX females (112, 131-133). Third, both the LH

surge event and *Kiss1* neurons in the AVPV/PeN are sexually dimorphic (favoring females), providing further correlative evidence for the role of AVPV/PeN *Kiss1* system in controlling the LH surge (115, 134). Specifically, unlike females, adult male rodents do not demonstrate LH surges even when treated with E_2 (135, 136). Correspondingly, males also have fewer *Kiss1* neurons and lower *Kiss1* expression levels in the AVPV/PeN than do females (114, 115). These findings suggest that higher numbers of AVPV/PeN *Kiss1* neurons in female rodents may comprise the underlying neural sex difference that generates the LH surge in females, but not males. In contrast, ARC *Kiss1* neurons, which are inhibited by E_2 treatment (111, 116, 131), are unlikely to play a significant role in generating the LH surge in rodents, though this may not be the case for some species such as sheep (137, 138).

Influence of the circadian system on kisspeptin neurons

If the LH surge is governed in part by *Kiss1* neurons in the AVPV/PeN, do these neurons also participate in the circadian component of the LH surge? Anatomically, *Kiss1* neurons in the AVPV/PeN are poised to mediate indirect circadian signaling to GnRH neurons, as the SCN sends axonal projections to the AVPV/PeN (76, 80), which itself projects to GnRH neurons (139). The most compelling evidence for an involvement of kisspeptin signaling in the circadian gating of the LH surge comes from recent findings that AVPV/PeN *Kiss1* neurons demonstrate robust circadian patterns of gene expression and neuronal activation, coincident with the circadian pattern of LH (133). Specifically, in a study by Robertson et al. (2009), female mice were entrained to a 14-10 light-dark cycle for several weeks, then OVX and implanted with an E_2 implant that produces constant, steady-state E_2 levels. This E_2 paradigm had previously been shown to produce a reliable circadian-timed LH surge each day around the time of lights off (140). On the day of the surgery, OVX+E_2 mice were then permanently transferred into constant darkness to remove changes in external light cues, which could potentially mask or induce circadian rhythmicity of *Kiss1* circuits. All mice were then sacrificed 2 days later at one of eight different circadian times throughout the day. Under these constant conditions, OVX+E_2 mice sacrificed an hour before or at the onset of subjective night [defined as circadian time (CT) 12] showed a robust surge of LH whereas low levels of LH were detected in the morning (CT 0, 4), early afternoon (CT 8), and late night (CT 20) (133). In synchrony with the circadian pattern of LH secretion, OVX+E_2 mice demonstrated a strong circadian pattern of *Kiss1* gene expression, with higher *Kiss1* mRNA levels in the AVPV/PeN (determined via *in situ* hybridization) at CT 11 and CT 12 than at earlier or later time points (e.g., CT 0, CT 4, and CT 20) (Figure 1) (133). In addition to circadian changes in *Kiss1* gene expression, AVPV/PeN *Kiss1* neurons also displayed a robust circadian pattern of *c-fos* co-expression, with peak *c-fos-Kiss1* co-expression occurring in synchrony with peak LH secretion, around CT 11 and 12 (133). These findings suggest that circadian-timed neuronal activation of *Kiss1* neurons in the AVPV/PeN is involved in the circadian induction of the preovulatory LH surge. However, this conclusion awaits definitive support from future experiments in which selective blockade of *Kiss1* neuronal activity in the AVPV/PeN at the presumptive time of the GnRH/LH surge prevents the surge from occurring.

Given the robust circadian pattern of activation of *Kiss1* neurons in OVX+E_2 mice, Robertson et al. (2009) also asked whether the typical absence of an LH surge in OVX females could reflect a lack of circadian activation of *Kiss1* neurons in the AVPV/PeN. The authors repeated their experiment in OVX mice that were not treated with E_2. Interestingly, no circadian changes in LH, *Kiss1* gene expression, or *Kiss1/c-fos* coexpression were detected in these OVX mice, with each of these parameters being low at all time-points (Figure 1) (133). This intriguing data suggests that the circadian activation of *Kiss1* neurons in the AVPV/PeN is dependent on the presence of elevated E_2, perhaps explaining why OVX females and diestrus females (with only low E_2) do not display late afternoon LH

surges. Similar findings have recently been reported in female hamsters, in which kisspeptin-Fos-immunoreactive co-expression in the AVPV/PeN displays circadian changes which are much weaker (though not eliminated) in OVX than OVX+E_2 hamsters (141). The reason for the slightly different results between OVX hamsters (dampened kisspeptin-Fos circadian rhythm) and OVX mice (eliminated *Kiss1*-Fos rhythm) is unknown, and may reflect a true species difference and/or technical differences (for example, in mice, *Kiss1* mRNA was measured whereas in hamsters, kisspeptin immunoreactivity was measured). Unfortunately, circulating E_2 levels were not measured in the OVX hamsters to verify that they were completely without E_2 at the time of analysis.

Because AVPV/PeN *Kiss1* neurons exhibit circadian patterns of gene expression and neuronal activation, it suggests these neurons receive circadian input from the SCN (though the possibility that *Kiss1* neurons are themselves autonomous circadian clocks has not been tested). Circadian input to *Kiss1* neurons in the AVPV/PeN may come from AVP neurons in the shell of the SCN. This conjecture is supported by recent immunohistochemical studies in female mice showing that AVP axonal projections arising from the SCN come in close proximity to AVPV/PeN *Kiss1* neurons, a finding confirmed at the electron microscopy level (142). Similar data has come from a recent hamster study in which AVP-containing fibers were found to appose AVPV/PeN kisspeptin cells (141). Importantly, in that study, lesioning the SCN abolished virtually all AVP appositions on kisspeptin neurons, suggesting that the source of the AVP fibers was in fact the SCN (141). Interestingly, in mice, the percentage of kisspeptin neurons apposed by SCN-derived AVP axons increases with E_2 treatment (~47% in OVX+E_2 vs. ~31% in OVX) (142), perhaps providing some explanation for the observed E_2-dependence of circadian regulation of the *Kiss1* system. Thus, AVPV/PeN *Kiss1* neurons may be gated by the SCN via AVP signaling. In support of this, the AVP receptor subtype V1a has been detected in AVPV/PeN kisspeptin neurons of female hamsters (141). In comparison, VIP connections to AVPV/PeN *Kiss1* neurons are far fewer in number and almost non-existent (< 1%, regardless of E_2 treatment), at least in mice (142).

Interestingly, the stimulatory effects of kisspeptin on GnRH neurons may also be circadian-gated (141). In hamsters, AVP administration increases c-Fos expression in AVPV/PeN kisspeptin neurons in both the morning and late afternoon, but c-Fos expression in GnRH neurons of the same animals is only upregulated by afternoon AVP treatment (141). This suggests that GnRH cells possess an intrinsic or extrinsic gating mechanism that temporally modulates their responsiveness to kisspeptin input, such that GnRH neurons are more sensitive to kisspeptin signaling in the late afternoon. What allows for this GnRH gating mechanism is unknown, but one possibility is a circadian regulation of the availability or signaling of Kiss1R in GnRH neurons. In support of this, GT1-7 cells (immortalized GnRH cells) show daily changes in the responsiveness to exogenous kisspeptin treatment (143). Furthermore, circadian clock proteins PER2 and BMAL1 demonstrate circadian rhythmicity within GnRH neurons *in vivo* (144), indicating that GnRH neurons possess the proper circadian machinery, at least in theory, to generate intrinsic circadian signals. VIP signals coming directly from the SCN may also play a role in timing the sensitivity of GnRH cells to incoming kisspeptin signals, though this remains to be tested. Additional support for changes in responsiveness to kisspeptin input comes from studies in rats and humans in which LH responses to kisspeptin administration differ with cycle stage, being more robust during the preovulatory phase (145, 146).

While the data above demonstrate the importance of the AVPV/PeN *Kiss1* system in regulating the LH surge, recordings of mouse brain slices curiously demonstrate a lack of response of AVPV kisppeptin neurons to AVP, VIP, or either antagonist, regardless of the stage of the mouse's estrous cycle (147). It must be determined if such insensitivity to SCN

neuropeptides is contingent on specific conditions, or if this is a difference between slice preparations and what occurs *in vivo*.

The RFRP-3 system: function and anatomy

It has been proposed that to fine-tune the excitability of GnRH neurons and the precise timing of the preovulatory LH surge, an inhibitory component complements the kisspeptin-driven stimulatory component. RFamide-related peptide (RFRP), the mammalian ortholog of the avian gonadotropin-inhibitory hormone (GnIH), modulates the reproductive axis by inhibiting GnRH neurons. Specifically, in mammals, the variant RFRP-3 has been implicated in GnRH neuronal inhibition. Administration of RFRP-3, both centrally and peripherally, leads to a rapid and significant inhibition of LH secretion, as first shown in rodents and later other species (148-150). Although there is some evidence suggesting that RFRP-3 acts in the pituitary, including the presence of the RFRP-3 receptor GPR147 in the pituitary (151), the inhibitory effects of RFRP-3 are likely achieved at the level of GnRH neurons. RFRP-3 treatment strongly decreases the electrical activity of GnRH cells in cultured brain slices, and RFRP-3 administration *in vivo* leads to suppressed c-Fos expression in GnRH neurons (152-154). Moreover, administration of RF9, an antagonist of the RFRP-3 receptor, leads to enhanced LH secretion in rats, further demonstrating the importance of RFRP-3 in regulating LH release (155) [though it should be noted that RF9 also antagonizes the actions of neuropeptide FF, in addition to RFRP-3, necessitating caution when interpreting these results].

RFRP-3-expressing neurons, found exclusively in the hypothalamic DMN of rodents, send numerous neural projections to GnRH cell bodies in the forebrain and GnRH axonal fibers in the median eminence; more than 40% of GnRH neurons receive RFRP-3 projections (148, 151). RFRP-3 neurons may mediate some aspect of estrogen negative feedback, as these cells express ERα and, in at least one study, showed increased c-Fos expression following E_2 administration (148). Since RFRP-3 is inhibitory to GnRH neurons, increased activity of RFRP-3 neurons might be predicted to result in increased inhibition of the reproductive axis, thereby mediating negative feedback. Alternatively, other evidence suggests that estrogen-mediated positive feedback may also involve changes in RFRP-3 neuronal system. Specifically, it has been hypothesized that RFRP-3 neurons are “turned off” around the time of the LH surge, thereby removing inhibition from GnRH neurons. This possibility is supported by recent findings of decreased RFRP-3 mRNA expression in the DMN of OVX+E_2 mice as well as decreased RFRP-3 cell numbers in proestrous versus diestrous hamsters, suggesting that elevated estrogen inhibits the RFRP-3 system (151, 156). In addition, the hamster study reported decreased Fos-RFRP-3 co-expression in proestrous females exhibiting an LH surge versus diestrous females. Such an E_2-mediated decrease in RFRP-3 signaling might allow for reduced inhibition of GnRH neurons, thereby magnifying the LH surge. However, this issue is controversial, as a similar study in rats showed no difference in RFRP mRNA levels of females that were OVX versus OVX+E_2 and P_4 (157). Furthermore, in OVX ewes, E_2 treatment does not significantly alter RFRP mRNA expression levels (158). Whether these discrepancies are due to species differences or other unaccounted factors has yet to be determined.

Linking the RFRP-3 system to the circadian clock

While RFRP-3 neurons send projections to GnRH neurons, they also receive projections from the SCN. However, while both AVP- and VIP-positive fibers from the SCN extend to the DMN in rodents (30, 151), it is not known which of these, if any, innervate RFRP-3 cells. Regardless, the presence of SCN-derived fibers in the DMN suggests that RFRP-3 neurons may play a role in the circadian component of positive feedback. In support of this, in female Syrian hamsters, RFRP-3 neurons display changes in protein expression levels

over the course of the day. Specifically, during the afternoon of proestrus (at the time of the LH surge), RFRP-3-immunoreactive neurons are reduced in number and also show reduced c-Fos coexpression, relative to earlier or later time-points (151). This circadian-timed decrease in the RFRP-3 system suggests a reduced inhibition by RFRP-3 on the GnRH system in the late afternoon. As with *Kiss1* neurons, E_2 is necessary for the temporal regulation of RFRP-3 neurons; without E_2, RFRP-3 neurons do not demonstrate daily changes in c-Fos expression (151). However, it remains to be determined how the SCN signals to RFRP-3 neurons and what the mechanism is underlying the E_2-dependence of this signaling. Moreover, since only a minority of RFRP neurons expresses ERα, circadian signals may play a more essential role in regulating RFRP-3 cells than hormonal signals (148).

Additional evidence for a role of RFRP-3 in positive feedback comes from an experiment where the circadian system of female hamsters was experimentally "split" into two separate circadian rhythms whose phases are 180 degrees (12 h) apart [using chronic exposure to constant light (159, 160)]. This "splitting" paradigm is known to cause each half of the bilateral SCN to functionally dissociate from the other and to continue running as a circadian clock with peak activity that is 12 h out of phase with the other half of the SCN. In this split condition, an E_2-treated female will exhibit 2 separate bouts of locomotor activity every day that begin ~12 h apart, along with 2 daily LH surges, with each surge occurring at the onset of one the two locomotor activity bouts (161). de la Iglesia et al. (162) showed that when one half of the SCN is active, the ipsilateral population of GnRH neurons is also active, with higher c-Fos co-expression than the contralateral GnRH population. Thus the two "split" LH surges each day are thought to be separately driven by the left and right GnRH populations which are activated ~ 12 h apart. Interestingly, Gibson et al. (2008) determined that the ipsilateral population of RFRP-3 neurons of split females shows lower activity than the contralateral RFRP population (151). Thus, the side of the brain in which GnRH neurons are being temporally activated is the same side where RFRP-3 neurons are concurrently less activated. This suggests that each side of the bilateral SCN may communicate to ipsilateral RFRP-3 neurons, which then signal to GnRH neurons.

Putting the Pieces Together: Current Model of the Circadian-Regulated Preovulatory LH Surge in Rodents

Clearly, many factors influence the rhythmic output of LH secretion, including multiple routes of circadian input to GnRH neurons as well as estrogen feedback to several areas of the hypothalamus and pituitary. While the neuroendocrine effectors kisspeptin and RFRP-3 have only recently been discovered, several studies have now implicated and expounded their roles in the circadian-timed preovulatory LH surge in rodents. At present, AVPV/PeN *Kiss1* neurons, which possess ERα, are thought to be key players in the positive feedback event, providing strong stimulatory input directly to GnRH neurons. In response to SCN-derived AVP signals or other yet-to-be defined circadian input, AVPV/PeN *Kiss1* neurons, which co-express the V1a receptor, exhibit robust circadian patterns of gene expression and *c-fos*-co-expression, peaking in the late afternoon/early evening. This circadian activation of AVPV/PeN *Kiss1* neurons is dependent on the sex steroid milieu, thereby allowing *Kiss1* neurons to serve as integrators of both circadian and E_2 signals that are each essential for the LH surge. The SCN also projects to DMN RFRP-3 neurons, which show circadian patterns of neuronal activation that are opposite to those of *Kiss1* neurons, being diminished in the late afternoon/early evening. However, unlike AVPV/PeN *Kiss1* neurons, it is not known which neurotransmitters the SCN uses to communicate with RFRP-3 cells, nor is it known if RFRP-3 cells express receptors for key SCN signaling factors. Moreover, the functional role of SCN-derived VIP projections, particularly those that direct target GnRH neurons, in the circadian-timed LH surge remains unknown. GnRH neurons directly integrate multiple

inputs from kisspeptin (stimulatory), RFRP-3 (inhibitory), and core VIP (likely stimulatory) neurons and may respond to some of these signals in a temporally-gated manner, resulting in a final coordinated output of high GnRH secretion that triggers the LH surge at a specific time. Table 1 summarizes changes in the expression levels of the main neuropeptides involved in regulating the timing and generation of the LH surge, and Figure 2 outlines our working model of the circadian and neuroendocrine network responsible for generating the preovulatory LH surge in rodents. This information will provide a framework for future experiments exploring the mechanism of the preovulatory LH surge in rodents and other species.

Despite the recent advances in our knowledge of the LH surge mechanism, several important questions still remain to be answered. Regarding both *Kiss1* and RFRP-3 neurons, does ERα oscillate in either population over the course of the day or between different stages of the estrous cycle? How does the SCN communicate with and regulate RFRP-3 neurons in the DMN? Do GnRH neurons exhibit circadian-dependent changes in sensitivity to RFRP-3 signals, as they appear to for kisspeptin, and do Kiss1R and GPR147 expression levels oscillate in a circadian manner in GnRH neurons? If these receptors do oscillate, are these temporal changes cell-autonomous or mediated by upstream SCN signaling? Lastly, do the RFRP-3 and *Kiss1* populations communicate with each other? Some RFRP-3 neurons project to the AVPV and, conversely, some kisspeptin fibers have been observed in the DMN (148), but this issue needs to be examined in more detail. Answering these questions will improve our understanding of the complexities that underlie the circadian basis of GnRH/LH secretion during E_2-medaited positive feedback, both in rodents and other species.

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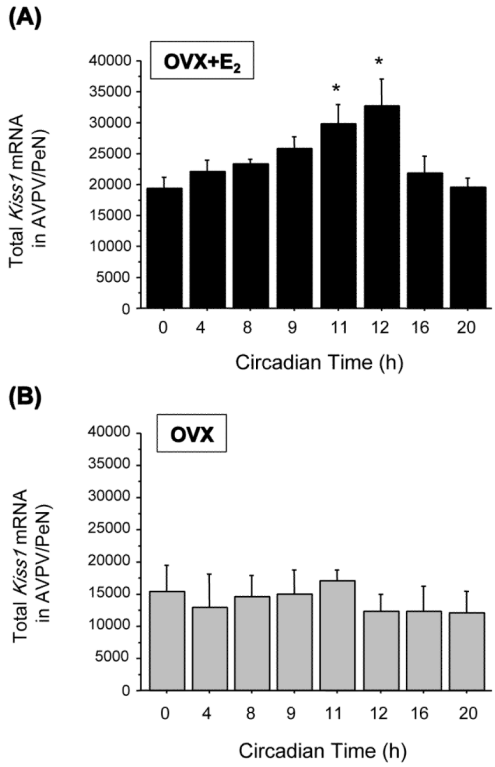

Figure 1.

Total *Kiss1* mRNA in the AVPV/PeN of OVX mice with (a) and without (b) E_2 treatment that were sacrificed at different circadian times. In the OVX+E_2 mice, an LH surge was detected at CT 11 and CT 12 (see Robertson et al., 2009). (*) indicates a statistical significance of $p<0.05$ relative to CT 0, CT 4, CT 8, and CT 20.

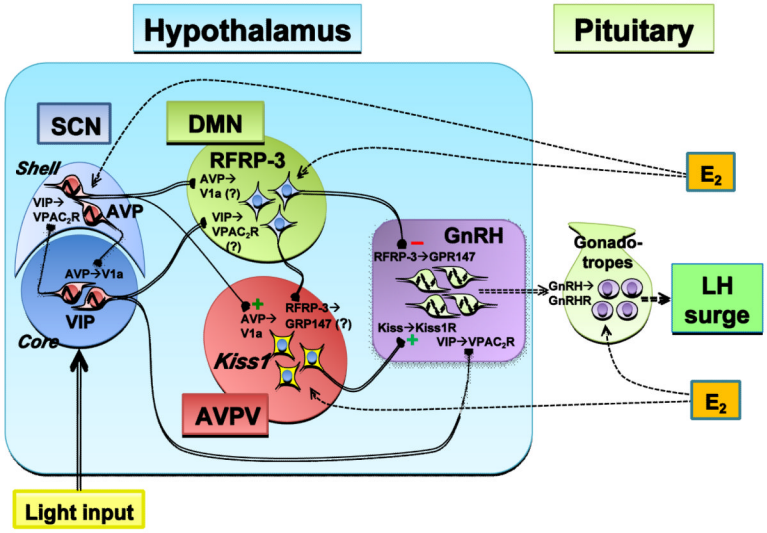

Figure 2.

Schematic summarizing the network of neurons and respective neuropeptides thought to participate in the regulation and timing of the female rodent preovulatory LH surge. This working model suggests that several levels of control are upstream of GnRH neurons: hormonal (ovarian-derived E_2), circadian (SCN-derived AVP and VIP), and intermediate activators/inhibitors (kisspeptin and RFRP-3). VIP neurons in the SCN project to the DMN and GnRH neurons, and AVP neurons in the SCN project to the DMN and AVPV/PeN *Kiss1* neurons, potentially providing circadian information relevant to the timing of the LH surge. *Kiss1* neurons directly stimulate GnRH neurons, while RFRP-3 neurons directly inhibit them. Some RFRP-3 neurons also project to the AVPV region, but it is not known if these projections target *Kiss1* neurons. GnRH neurons integrate input from *Kiss1*, RFRP-3, and VIP neurons directly and may respond to some of these signals in a temporally-gated manner. In addition, GnRH neurons may possess endogenous circadian oscillators, the function of which is unknown. See text for more detailed description and explanation of abbreviations.

Key: Solid lines with boutons represent neuronal connections, dotted lines with arrowhead represent hormonal pathways; Solid sinusoid and dotted sinusoid represents cell-autonomous oscillatory activity and potential cell-autonomous oscillatory activity, respectively.

Table 1

Summary of mRNA expression levels of neuropeptides potentially involved in the regulation of the preovulatory LH surge in female rodents, as well as corresponding data on the status of GnRH neuronal activation (Fos in GnRH) and the presence of an LH surge at different times of day (AM versus PM). Where no mRNA data is available, data for protein (immunoreactivity measures) is given instead and noted.

Neuropeptide (region)	OVX		OVX+E_2		Proestrus		References
	AM	PM	AM	PM	AM	PM	
AVP (SCN)	High	Low	High	Low	High	Low	(90, 163-166)
VIP (SCN)	Low	High/ Low*	Low	High	Low	High	(42, 90, 164, 165, 167)
Kiss1 (AVPV)	Low	Low	Med	High	Med	High	(131-133)
RFRP-3 (DMN)	Med/ High	Med/ High	High	High/ Low*	High (protein)	Med (protein)	(151, 156, 157)
Fos in GnRH	Low	Low	Low	High	Low	High	(64, 83, 84, 112, 168, 169)
LH surge	No	No	No	Yes	No	Yes	(7, 9, 62, 63, 70, 132, 133, 140, 151)

* conflicting data, see (90, 167) for VIP and (156, 157) for RFRP-3.