Published in final edited form as:

Adv Exp Med Biol. 2013 ; 784: 385–410. doi:10.1007/978-1-4614-6199-9_18.

Circadian Regulation of Kisspeptin in Female Reproductive Functioning

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Abstract

Female reproductive functioning requires the precise temporal organization of numerous neuroendocrine events by a master circadian brain clock located in the suprachiasmatic nucleus. Across species, including humans, disruptions to circadian timing result in pronounced deficits in ovulation and fecundity. The present chapter provides an overview of the circadian control of female reproduction, underscoring the significance of kisspeptin as a key locus of integration for circadian and steroidal signaling necessary for the initiation of ovulation.

Introduction

The circadian timing system universally coordinates central and peripheral physiology, providing temporal structure to homeostatic regulation and ensuring that physiological processes are maintained within optimal operating limits given changing demands over the course of day and night. Because hormones enter the general circulation, these signaling molecules have widespread influence over physiology and behavior, and represent ideal communicators of timing information. As a result, it is important to understand the means by which circadian rhythms in endocrine secretions are generated and the functional consequences of this temporal communication for downstream target systems.

Converging lines of evidence indicate a critical role for circadian timing in successful female reproduction across mammalian species, including humans. Women with irregular work or sleep cycles, for example, exhibit reduced fertility [1] and an increased spontaneous abortion rate [2], suggesting the importance of circadian functioning in ovulation and pregnancy maintenance. Likewise, the luteinizing hormone (LH) surge that initiates ovulation occurs in early morning in women [3] and diurnal rodents [4], but in early evening in nocturnal rodents (reviewed in [5]). In rodents, disruptions of the master circadian clock in the brain, its neural output, or the genes regulating cellular clock function lead to pronounced abnormalities in ovulation and fecundity [6–8]. Given the necessity of proper hormonal timing in female reproductive health, and the experimental tractability of the reproductive axis, the study of female reproductive functioning represents an ideal model

system for understanding circadian endocrine control. As is apparent throughout this book, kisspeptin is essential for reproductive functioning and, not surprisingly, represents a critical node in the network of circadian regulation underlying female reproductive health. The present chapter provides a broad overview of circadian control of female reproduction, underscoring the significance of kisspeptin signaling within this framework.

The Circadian Timing System

Four decades ago, two studies provided strong evidence that the suprachiasmatic nucleus (SCN) of the hypothalamus was the locus of the master circadian pacemaker in mammals [9, 10]. In these initial studies, electrolytic lesions of the SCN abolished rhythms in locomotor and drinking behavior and adrenal glucocorticoids, suggesting that either the circadian clock is localized to the SCN, that the SCN is part of a larger network responsible for circadian rhythm generation, or that fibers of passage required for circadian functioning traveled through this neural locus. Numerous converging lines of evidence since these initial investigations, from a host of laboratories, have confirmed the role of the SCN as a master pacemaker. For example, transplants of donor SCN tissue into the brains of arrhythmic, SCN-lesioned hosts restore circadian rhythmicity in behavior [11, 12]. Importantly, rhythms are restored with the period of the donor SCN, indicating that the transplanted tissue does not act by restoring host-brain function but that the “clock” is contained in the transplanted tissue. Furthermore, circadian rhythms in neural firing rate persist in isolated SCN tissue maintained in culture [13], demonstrating that input from extra-SCN brain sites is not necessary for circadian rhythm generation. Although circadian rhythms are endogenously generated, in order to be adaptive for an organism, these rhythms must be synchronized to the external environment. This entrainment is accomplished via direct neural projections from intrinsically photosensitive retinal ganglion cells to the circadian clock in the SCN [14–19].

At the cellular level, circadian rhythms are generated by 24-h autoregulatory transcriptional/translational feedback loops consisting of “clock” genes and their protein products (Fig. 18.1) [20–23]. In mammals, the feedback loop begins in the cell nucleus where CLOCK and BMAL1 proteins heterodimerize and drive the transcription of the Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry2*) genes by binding to the E-box (CACGTG) domain on their gene promoters. Once translated, PER and CRY proteins build in the cytoplasm of the cell over the course of the day, and inevitably form hetero- and homodimers that feed back to the cell nucleus to inhibit CLOCK:BMAL1-mediated transcription. The timing of nuclear entry is balanced by regulatory kinases that phosphorylate the PER and CRY proteins, leading to their degradation [21, 24, 25]. Two other promoter elements, DBP/E4BP4 binding elements (D boxes) and REV-ERB α /ROR binding elements (RREs) [26], also participate in cellular clock function. REV-ERB α , an orphan nuclear receptor, negatively regulates the activity of the CLOCK:BMAL1. The same mechanism controlling *Per* and *Cry* gene transcription also controls transcription of REV-ERB α . Similarly, the transcription factor DPB is positively regulated by the CLOCK:BMAL1 complex [27] and acts as an important output mechanism, driving rhythmic transcription of other output genes via a PAR basic leucine zipper (PAR bZIP) [28].

Historically, given the fundamental role of the SCN in maintaining rhythmicity, it was believed that only cells in this nucleus are capable of intrinsic rhythm generation. However, this view was challenged in a seminal study establishing that cultured rat fibroblasts exhibit oscillations in core clock genes when presented with a serum shock [29]. Over the next decade, it became clear that oscillations are a pervasive property of cells throughout the CNS and periphery. However, in the SCN, individual oscillators are coupled to form a coherent network where the phase relationship of individual oscillators is coordinated. In other central and peripheral systems, whereas individual cells are competent oscillators, without SCN communication, the phase relationship among populations of oscillators is lost, leading to an abolition of tissue-level rhythmicity [30]. Thus, it is now more appropriate to conceptualize the “circadian system” as an assembly comprised not only of a master clock, but also a series of subordinate clocks whose phase and activity is coordinated by the SCN. As described further below, this hierarchy of circadian control has important implications for understanding the means by which kisspeptin initiates the LH surge and ovulation.

Circadian Control of the Preovulatory LH Surge and Ovulation

Discovery of Ovulatory Circadian Control

The neural mechanisms regulating ovulation are under circadian control in many spontaneously ovulating species, ensuring that the timing of maximal fertility is concomitant with the period of highest sexual motivation [31, 32]. Superimposed upon this circadian control is a dependence of the reproductive cycle on estradiol to ensure proper maturation of the oocyte at the time of ovulation. The notion that rodent ovulation is under circadian control was first proposed based on data from a classic study by Everett and Sawyer [33]. In this seminal paper, Everett and Sawyer sought to determine if ovulation required “neurogenic” activation of the reproductive axis. To explore this possibility, rats were injected with barbiturate to inhibit neural communication at the time of ovulation. Through a series of studies, they determined that a single barbiturate injection delayed ovulation by 24 h, despite the fact that the impact of the drug was short-lived. Based on this observation, the authors proposed that a 24 h neural signal initiates ovulation.

Over two decades following the work of Everett and Sawyer, Zucker and colleagues performed an elegant series of studies providing further evidence for a 24 h clock in ovulation and associated sexual motivation in Syrian hamsters (*Mesocricetus auratus*). When held in a light: dark (LD) cycle, ovulation and the onset of behavioral receptivity occur precisely every 96 h in this species [34]. This rhythm in reproductive activity is endogenously generated and persists in constant conditions with a period four times their free-running circadian period. Exposing hamsters housed in constant darkness (DD) to deuterium oxide (a treatment that results in a lengthening of the free-running period), resulted in a lengthening of the ovulatory cycle to precisely four times the period of an individual's circadian rhythm, establishing that the ovulatory cycle and locomotor rhythms are governed by a comparable endogenous timing system [35]. Because estrous and activity onset were coupled temporally, it was suggested that the LH surge and locomotor activity are controlled by either a single, endogenous oscillator or a coupled, multioscillator system that regulates the rhythms of each process independently [35]. The former hypothesis

postulated that the reproductive axis “tracks” four circadian cycles and ovulation occurs after the count is complete.

Converging lines of evidence over the next three decades established that both of these hypotheses are partially correct. We now know that the SCN provides a daily, stimulatory signal to the reproductive axis each day of the estrous cycle, closely preceding the active phase, in most spontaneously ovulating rodents [5, 36, 37], indicating that a single clock subserves both processes. However, this signal is only effective at stimulating the GnRH system to produce the LH surge in the presence of estradiol concentrations above a critical threshold. Prior to the day of proestrus, the developing ovarian follicles secrete insufficient estradiol to fulfill this criterion. The nature of the daily stimulatory signal from the SCN can be unmasked by implanting animals with estradiol capsules that result in proestrus concentrations of this hormone; in this case, daily LH surges occur [37–39]. Regarding the second hypothesis suggesting a multioscillator organization, although distinct clocks do not underlie locomotor rhythms and estrus, a hierarchical clock structure exists in which the SCN acts as the master pacemaker coordinating rhythmicity in subordinate oscillator systems of the reproductive axis, an arrangement discussed further below.

Sex Steroid and Circadian Integration in Ovulatory Control

Through negative-feedback effects of sex steroids, LH is maintained at low concentrations throughout most of the ovulatory cycle, paradoxically, high concentrations of estradiol are required for the SCN to trigger ovulation (i.e., positive feedback) [5, 36, 37, 40]. The site(s) of integration for positive and negative-feedback effects of estradiol with the circadian timing system are complex and not fully understood. As described below, current evidence indicates that one important site of integration for the positive feedback effects of estradiol with circadian signaling is the kisspeptin network in the anteroventral periventricular nucleus (AVPV).

Circadian Neurochemical Communication and Ovulation

Despite the fact that the SCN is crucial for both locomotor behavior and the initiation of ovulation, each process is likely mediated by distinct communication modalities. In arrhythmic, SCN-lesioned hamsters, fetal SCN transplants restore locomotor, but not endocrine, rhythms [41–43]. Restoration of locomotor and other behavioral rhythms occurs in the absence of neural outgrowth from the grafted SCN, suggesting that intact neural connections are required for endocrine rhythms, whereas behavioral rhythms can be supported by a diffusible signal. Perhaps the best evidence indicating that the SCN communicates via neural connections to the GnRH system to initiate the LH surge comes from studies using hamsters with “split” activity rhythms [44]. When housed in constant light, some hamsters exhibit a splitting in behavior, with two daily activity bouts separated by 12 h, each reflecting an antiphase oscillation of the left and right sides of the bilateral SCN [44–46]. Under these circumstances, ovariectomized (OVX) hamsters treated with estradiol exhibit two LH surges in a 24-h period, each phase-locked to an individual activity bout [47]. Because the SCN communicates principally ipsilaterally [48–51], the authors hypothesized that, if a neural output signal from the SCN initiates the GnRH/LH surge, then one hemispheric set of GnRH neurons should be activated, ipsilateral to the activated SCN,

with each locomotor activity bout. Conversely, if controlled by a diffusible signal, then the GnRH system should be activated concurrently on both sides of the brain, twice daily, 12 h apart [52]. Under these split conditions, the former possibility manifested, confirming the importance of neural SCN communication to the GnRH system in ovulatory control. As described below, more recent studies by this group and others establish that SCN communication to kisspeptin cells in the AVPV underlies, in part, this neural signaling cascade initiating the GnRH/LH surge [53–56].

Direct SCN Signaling to the GnRH System

The SCN communicates both monosynaptically and multisynaptically to the GnRH system. Whereas much progress has been made in understanding both modes of communication, most studies to date have investigated the contribution of individual pathways in isolation, making it difficult to ascertain the significance of interactions between these pathways. One SCN neuropeptide that has received considerable attention as a modulator of ovulation is vasoactive intestinal polypeptide (VIP). Neurons synthesizing VIP are located in the retinorecipient, ventrolateral SCN “core” [57–59], and project monosynaptically to GnRH neurons [49, 60] that express the VIP receptor VPAC₂ [61]. SCN-derived VIP input to GnRH neurons is sexually dimorphic, with female rats exhibiting higher VIPergic innervation [60], suggesting a critical role in female reproductive functioning. Likewise, the number of VIP-GnRH contacts increases during the post-pubertal transition to reproductive competence [62], a time when estradiol positive feedback is first established. VIP-innervated GnRH neurons exhibit lower activation levels in middle-aged female rats, suggesting that the degradation of this signaling cascade participates in the transition to reproductive senescence in female rodents [63]. In reproductively competent females, GnRH neurons receiving VIPergic input preferentially express the neural activation marker, FOS, at the time of the LH surge [64]. In vivo antisense antagonism of VIP production in the SCN abolishes GnRH/FOS activation in female rats, providing further support for the necessity of VIP output in surge generation [65, 66]. Finally, blocking the VPAC₂ receptor attenuates GnRH neuronal cell firing during the afternoon surge in female, estradiol-treated mice [67]. Together, these lines of evidence suggest that direct VIP projections from the SCN to the GnRH system positively drive the GnRH/LH surge. The potential means by which this pathway synergizes with multisynaptic projections from the SCN to the reproductive axis is discussed further below.

Indirect SCN Signaling to the GnRH System

Historically, it was thought that estradiol positive feedback occurred through the convergence of estrogenic and circadian signaling at the level of GnRH neurons. The observation that GnRH neurons do not express estrogen receptor α (ER α), the estrogen receptor subtype mediating the positive feedback effects of estradiol [68–70], motivated the search for additional neural loci at which stimulatory circadian and estrogenic signals converge. The AVPV emerged as a likely site as neurons in this brain region send monosynaptic projections to GnRH cells, express FOS coincident with the LH surge, and lesions of the AVPV eliminate estrous cyclicity in both intact and OVX, estradiol-treated rats [8, 71–74]. Moreover, the SCN sends pronounced monosynaptic projections to cells in

the AVPV that express ER α [68, 75–77], leading to the search for the SCN neurochemical cell phenotypes sending projections to the AVPV.

Vasopressinergic (AVPergic) cells in the dorsomedial SCN target ER α —expressing cells in the AVPV [50, 75, 76, 78–80], and AVP injections produce surge-like LH levels in SCN-lesioned, OVX, estradiol-treated rats [81]. Likewise, cells in this brain region express the vasopressin receptor, V1 $_a$ [82, 83]. Antiestrogens targeting the AVPV inhibit the LH surge in OVX, estradiol-treated rats [84], confirming the importance of estrogen signaling in ovulation. Vasopressin gene transcription in the SCN is directly controlled by the molecular clockwork at the cellular level [85–87] and is released in a circadian manner [88], with a peak coinciding with the onset of the LH surge [89, 90]. AVP release is synchronous with GnRH secretion in cocultures of medial preoptic area (mPOA) and SCN brains slices [91]. By contrast, central AVP receptor antagonists attenuate the LH surge in proestrous rats [92]. Finally, the inability of *clock* mutant mice to generate an LH surge is associated with diminished *AVP* mRNA expression in the SCN, a phenotype that can be restored via central injections of AVP, further linking this peptide to the circadian control of ovulation [6].

In rodents, *Kiss1* mRNA expressing cells in the AVPV and arcuate (ARC) nuclei exhibit robust ER α labeling [93–97]. The effects of estradiol on kisspeptin activity, however, varies by nucleus, with ovariectomy decreasing *Kiss1* mRNA in the AVPV and increasing *Kiss1* expression in the ARC, pointing to a role for kisspeptin in estradiol positive and negative feedback, respectively [94, 98]. Exogenous kisspeptin administration potently induces LH release as well as upregulates FOS expression in GnRH neurons [93, 99–102]. One recent study used genetic ablation strategies to explore the importance of kisspeptin in puberty and adult reproductive functioning. Genetic ablation of kisspeptin cells or cells expressing GPR54 throughout development does not impact female puberty onset or fertility in adult animals, although loss of GPR54-expressing cells results in blunted LH, reduced ovarian weights, and irregular estrous cycling [103]. In adult animals, acute ablation of kisspeptin neurons markedly disrupts fertility and estrous cyclicity, whereas removal of ~93% of GPR54-expressing GnRH cells results in more mild reductions in LH, fertility, and estrous cycling [103]. Together, these findings suggest that kisspeptin signaling is required for adult female reproductive functioning and compensatory mechanisms can overcome the necessity for kisspeptin when this gene is inactivated throughout development. However, given that as few as three GnRH neurons are sufficient to support activity of the HPG axis [104, 105], it is unclear whether GPR54-expressing GnRH neurons can be dispensed with in LH surge control if a few GPR54-expressing cell remain following the ablation.

The observation across rodent species that the SCN projects to the AVPV, and that this brain region is essential for production of the LH surge, combined with the knowledge that AVPV kisspeptin cells respond positively to estradiol, made these cells an attractive target of exploration in the initiation of GnRH/LH surge. An early study showing that *Kiss1* cells in the AVPV express FOS at the time of the LH surge in naturally cycling, OVX, estradiol-treated rats [106, 107], provided strong support for this possibility. To explore the circadian control of this activation pattern, Kauffman and colleagues maintained mice in constant conditions and examined *Kiss1* mRNA and the percentage of *Kiss1* cells expressing the *c-fos* gene (Fig. 18.2). The maintenance of a circadian pattern in the absence of environmental

cues would suggest endogenous rhythmic control through a circadian mechanism rather than a rhythm driven by environmental time cues. The authors established that the circadian pattern of *Kiss1* expression and the percentage of *Kiss1/c-fos* cells persist in constant darkness [56], with peak expression of both measures coordinated with the LH surge, suggesting endogenous circadian regulation of this cell population rather than reliance on external temporal cues. This daily pattern of *Kiss1* expression, and *Kiss1* cells expressing *c-fos*, is abolished by ovariectomy, and reinstated following steady-state estradiol replacement [56], indicating a permissive role for estradiol in the circadian control of *Kiss1* in this species.

These results point to either an endogenous, self-sustained rhythm in kisspeptin neurons, circadian control through upstream projections from the SCN, or a combination of both mechanisms of control. We examined these possibilities in Syrian hamsters. Consistent with findings in mice, FOS expression in kisspeptin immuno-reactive (ir) cells expressed a daily rhythm in OVX, estradiol-treated hamsters, with peak coexpression concomitant with the timing of the LH surge. In contrast to results observed in mice, ovariectomy results in a blunted rhythm of kisspeptin-FOS coexpression, but not its abolition [54]. These latter findings point to potential species differences in the role that estrogen plays in AVPV kisspeptin regulation and/or posttranscriptional modification of the *Kiss1* gene, resulting in differences in cells visualized with mRNA versus protein analyses. To determine whether the SCN projects to kisspeptin cells to mediate these observed rhythms, we examined projections from VIPergic and AVPerigic SCN cells, given the role of these neuropeptides in positively driving the LH surge. We found that AVPerigic SCN cells project directly to a majority of kisspeptin-ir cells, whereas VIPergic SCN cells did not (Fig. 18.3). In mice, AVPerigic projections to AVPV kisspeptin cells have also been identified, with synapses confirmed at the electron microscopy level, suggesting a common mechanism of circadian control across species [108]. Additionally, these authors established that estrogen increases the percent of GnRH cells with AVPerigic terminal appositions, providing an additional mechanism by which estrogen integrates with circadian signaling to stimulate kisspeptin neurons at the time of ovulation [108].

More recently a creative series of studies by de la Iglesia and colleagues further established the specific means by which the SCN regulates *Kiss1* expression and the LH surge [53]. First, to determine if the SCN communicates ipsilaterally to *Kiss1*-expressing neurons in the AVPV, and to assess the significance of this communication for GnRH cell activation at the time of the surge, the authors lesioned one half of this nucleus and examined AVPV *Kiss1* expression and FOS expression in GnRH cells in female rats (Fig. 18.4). Both *Kiss1* expression and the percentage of GnRH cells expressing FOS were reduced on the side of the brain ipsilateral to the lesion, suggesting that the SCN communicates ipsilaterally to AVPV kisspeptin cells and that this stimulation is necessary for activation of the GnRH system. By using a lighting regimen previously established by this group to desynchronize rhythms in the dorsomedial and ventrolateral SCN [109], the authors show that the dorsomedial SCN oscillates independently of the LD cycle and that both AVPV *Kiss1* expression and the LH surge are coupled to oscillations in this SCN subregion (Fig. 18.5) [53]. Together with previous data supporting a role for AVPerigic circadian control of AVPV

kisspeptin neurons [54–56, 110], these data provide strong evidence for an important role of this indirect SCN-kisspeptin circuit in initiating the GnRH/LH surge to stimulate ovulation.

Previous findings indicate that that administration of AV P can only induce the preovulatory LH surge within a narrow time window [111], suggesting a gated mechanism of control at SCN target loci. The gating of SCN information flow may be controlled within kisspeptin cells in the AVPV, at the level of GnRH neurons, or a combination of both mechanisms. To select among these possibilities, we examined whether (1) kisspeptin cells within the AVPV respond in a time-dependent manner to AV P stimulation and (2) if GnRH neurons display time-dependent sensitivity to kisspeptin signaling. If time-dependent sensitivity is controlled at the level of the AVPV, then one would expect kisspeptin cells to exhibit daily changes in sensitivity to AV P stimulation and contain an endogenous time-keeping mechanism.

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 AV P and kisspeptin administration. Our findings indicate that the kisspeptin system responds indiscriminately to AV P administration, regardless of time of day, whereas the GnRH system is only sensitive to kisspeptin stimulation at the time that the surge would normally occur (Fig. 18.6) [54]. These results further support the notion that kisspeptin cells do not keep circadian time but, instead, that their activity is driven by AVPergic SCN cells. However, these findings point to an important role for autonomous circadian oscillators in GnRH cells underlying time-dependent sensitivity to upstream signaling. Alternatively, it is possible that the master clock in the SCN communicates timing information to GnRH cells that do not maintain the capacity for endogenous rhythmicity. Given that SCN-derived VIPergic cells project monosynaptically to GnRH neurons, this cell phenotype represents an ideal candidate to communicate such timing information. Finally, a combination of both mechanisms may underlie such daily changes in GnRH cell sensitivity, with VIPergic SCN communication synchronizing independent GnRH cellular oscillators to coordinate the timing in their responsiveness to upstream signaling.

Clock Genes and Kisspeptin Control of Ovulation

The same clock genes that drive circadian rhythms at the cellular level in the SCN are also expressed throughout other brain regions and peripheral tissues. GnRH cells express clock genes, both in vitro [112, 113] and in vivo [114, 115]. Mice with a mutation in the essential circadian clock gene, *Clock*, display abnormal estrous cycles and abnormal LH surge induction in response AVP administration [6], suggesting an important role for clock genes (potentially in GnRH cells) in normal estrous cycling and ovulation. Studies in which the rescue of *Clock* is restricted to the SCN of these mice would help to clarify whether circadian disruption at the level of the SCN or GnRH neurons is responsible for the deficits observed in these animals. Consistent with our findings in vivo, immortalized GnRH neurons (i.e., GT1-7 cells) exhibit ~24 h changes in sensitivity to kisspeptin and VIP signaling [113]. These findings suggest that an endogenous timing mechanism in GnRH cells gates daily changes in responsiveness to upstream, stimulatory neurochemicals, at least under these culture conditions. More recently, Chappell and colleagues found that estrogen-treated GT1-7 cells exhibit a rhythm in *GPR54* expression that is abolished in the absence of estrogen [116]. These results are intriguing given the absence of ER α in this cell population,

suggesting participation of ER β in this rhythm of *GRP54* transcription. Together with our findings in vivo [54], these findings indicate that GnRH cells maintains the ability to oscillate independently, potentially as a mechanism gating responsiveness to signals initiating ovulation. Whether or not SCN communication to the GnRH system is necessary to maintain phase coherence among this population of cells represents an important question for future enquiry.

Circadian Control of Estrogen Negative Feedback

Most work to date on the circadian control of ovulation has focused on estrogen positive feedback. Few studies have explored the neurochemical substrates and target loci at which circadian and estrogenic signals converge to maintain LH at low concentrations throughout the majority of the estrous cycle (i.e., negative feedback), and the mechanisms that suppress negative feedback at the time of the LH surge. Because estrogen suppresses kisspeptin activity in the Arc [94, 98], this population of cells is thought to be an important component of estradiol negative feedback. Whether the SCN projects to kisspeptin cells in the Arc has not been explored and represents an important potential mechanism by which suppression of positive drive to the reproductive axis might be coordinated by the circadian system prior to the LH surge.

We explored whether a neuropeptide in the same family as kisspeptin, gonadotropin-inhibitory hormone (GnIH; also known as RFamide-related peptide- 3), participates in circadian-controlled estradiol negative feedback. In 2000, Tsutsui and colleagues isolated a dodecapeptide from Japanese quail brain that dose- dependently inhibits gonadotropin release from cultured quail pituitary that they named GnIH [117]. GnIH acts as a pronounced negative regulator of HPG axis activity in all species investigated, including hamsters, mice, rats, cattle, sheep, nonhuman primates and humans (reviewed in [118 – 121]). Recently, one manuscript showed that GnIH leads to increased LH secretion in male hamsters, suggesting that it is important to consider sex and physiological state when investigating the impact of this neuropeptide [122]. In rodents, GnIH cell bodies are localized to the dorsomedial hypothalamus (DMH) with projections forming close appositions to GnRH cells [123, 124], suggesting the potential for direct, neural regulation of the GnRH network. Additionally, recent work in mice and Siberian hamsters indicates that a marked number of GnRH neurons express GnIH receptor [125, 126]. Whether or not endogenous GnIH acts on the pituitary, in addition to GnRH cells, in mammalian species requires further investigation to clarify discrepant findings across studies and species [127– 131].

We first examined the possibility that GnIH participates in mediating the negative- feedback effects of estradiol. Treatment of female rats with GnIH results in marked inhibition of GnRH neuronal activity at the time of the LH surge, providing support for this possibility [132]. In Syrian hamsters, we found that GnIH-ir cells express ER α and respond to acute estradiol treatment with increased FOS expression, suggesting activation by gonadal steroids [123]. Contrasting results were observed in one recent report, with treatment of mice with estrogen for 4 days leading to a decrease in GnIH mRNA expression [133]. The discrepancy in the impact of estrogen in mice and hamster may result from the timing at which the brains

were collected for analysis and the dose/duration of estrogen treatment. Both of these possibilities represent interesting areas of investigation that will help to clarify the specific role of this neuropeptide in female reproductive axis regulation.

We next explored whether GnIH neurons might be a locus of integration for steroidal and circadian signals, providing a mechanism to coordinate the removal of estradiol negative feedback with SCN-mediated stimulation of the GnRH/LH surge. First, we examined the pattern of GnIH cellular activity, uncovering a daily pattern with trough activity at the time of the LH surge, suggesting the removal of negative feedback at this time [127].

Additionally, using anterograde tract tracing, we found that the SCN projects to a large proportion of GnIH cells, providing a mechanism for timing removal of negative drive on the GnRH system (Fig. 18.7). Finally, by exploiting the “splitting” phenomenon seen in hamsters housed in LL described previously, we found that activation of the GnIH system is asymmetrical. Importantly, this asymmetry is opposite to that seen for the GnRH system, suggesting that the SCN concomitantly activates ipsilateral GnRH cells at the same time as removing the suppressive influence of GnIH on the same side of the brain (Fig. 18.7) [127]. Whether or not removal of negative feedback is required for the LH surge requires further empirical investigation. A recent series of studies by Pineda et al. using a selective antagonist (RF9) of GnIH provides converging evidence for a role of GnIH in the LH surge, with injections of RF9 increasing LH throughout the ovulatory cycle [134]. These findings are consistent with a role for GnIH in maintaining low LH concentration throughout the ovulatory cycle. Together, these findings and those for kisspeptin, point to an important role for these neuropeptides in the integration of positive and negative effects of estradiol with circadian signaling in the generation of the GnRH/ LH surge.

Circadian Implications for Reproductive Aging

Age-related decline in reproductive axis function is common across species, with initiation of this decline typically occurring midway through life. In female rodents, this waning in reproductive function occurs, at least in part, from a reduction in the ability of the SCN to stimulate the neural circuits underlying ovulatory functioning. In middle-aged hamsters, for example, the peak level of LH is delayed and exhibits a reduced amplitude relative to young, reproductively healthy animals [135]. These changes are associated with deficits in GnRH cell activation. In regularly cycling young females, 34–40% of GnRH neurons express immediate early genes on the afternoon of proestrus compared to 9–14% in middle-aged animals; the total number of GnRH-immunoreactive neurons does not differ between the two groups [136]. This finding suggests that the SCN may not provide adequate stimulation to the GnRH system in aged animals. In support of this possibility, VIP mRNA, but not AVP mRNA, becomes arrhythmic in the SCN of middle-aged female hamsters [137], and suppression of SCN VIP in young female hamsters results in accelerated deficits in GnRH activation and an LH surge that mimics that of an aged population [65,66]. Together, these findings suggest that age-related deficits in the neuroendocrine mechanisms mediating ovulation may result from loss of function at the level of the SCN.

Whether or not alterations in circadian signaling to the kisspeptin system contribute to reductions in reproductive competence with age has not been directly explored. However,

two recent studies provide suggestive evidence for this possibility [138, 139]. In middle-aged female rats, *Kiss1* mRNA and protein are reduced at the time of estradiol positive feedback relative to young animals, whereas kisspeptin injections into the preoptic area rescue LH release and surge amplitude [138, 139]. These findings are consistent with the notion that reduced circadian drive to kisspeptin neurons participates in aging-associated reductions in LH surge function. Alternatively, it is possible that deficits in estradiol signaling to AVPV kisspeptin cells with advancing age account for reductions in estradiol-induced kisspeptin activity. Whether either or both of these possibilities account for ovulatory decline with advancing age requires further exploration. Nonetheless, given these recent findings, it is noteworthy that kisspeptin not only represents a key neuropeptide initiating the onset of reproduction, but may also be a critical player in the transition to reproductive senescence, further pointing to a prominent role for this neuropeptide in maintaining reproductive competence.

Conclusions and Considerations

Given the importance of circadian timing in female reproductive functioning and health, and the key role played by kisspeptin in the circadian network driving ovulation, it is critical that a full understanding of the specific mechanisms and neurochemicals pathways driving the timed production and secretion of this neuropeptide be garnered. The present overview points to the SCN as a central clockwork driving the coordinated activity of a host of positive and negative regulators of the reproductive axis (Fig. 18.8). Future studies in which circadian signaling to relevant nodes downstream of the SCN, as well as circadian timing in these targets loci, is manipulated through pharmacology and molecular/cellular biology will help to further establish, and clarify, the specific role played by this circadian timing hierarchy in female reproduction. For example, does GnRH exhibit time-dependent specificity to all modulatory neurochemicals, only kisspeptin, or only positive regulators of GnRH activity? Likewise, in addition to estrogen signaling, what other positive and negative regulators of the reproductive axis converge on kisspeptin cells to integrate with circadian signaling and precisely time the GnRH/LH surge? Recent evidence in mice suggests that at least GnIH acts directly on a subset of kisspeptin cells [125], providing a mechanism for kisspeptin cell inhibition prior to initiation of ovulation and sexual motivation. Additionally, it is unclear why the SCN projects both directly to the GnRH and kisspeptin systems to positively drive GnRH secretion when integration at a single locus is more parsimonious. Finally, most studies to date investigate GnRH secretagogues (e.g., GABA, dynorphin, glutamate) in isolation or pairs. Future studies applying multivariate connectomic, proteomic, and genomic analyses to understand the complex interactions among these systems, and their integration with circadian control, will be necessary to gain a full understanding of the complexity underlying normal female reproduction and apply this knowledge to disorders of puberty and infertility.

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Fig. 18.1.

A simplified model of the intracellular mechanisms responsible for mammalian circadian rhythm generation. The process begins when CLOCK and BMAL1 proteins dimerize to drive the transcription of the *Per* (*Per1* and *Per2*) and *Cry* (*Cry1* and *Cry2*) genes. In turn, *Per* and *Cry* are translocated to the cytoplasm and translated into their respective proteins. Throughout the day, *PER* and *CRY* proteins rise within the cell cytoplasm. When levels of *PER* and *CRY* reach a threshold, they form heterodimers, feed back to the cell nucleus, and negatively regulate CLOCK: BMAL1-mediated transcription of their own genes. This feedback loop takes approximately 24 h, thereby leading to an intracellular circadian rhythm. See text for additional details

Fig. 18.2.

(a) Representative photomicrographs of Kiss1 mRNA and c-fos mRNA coexpression in the AVPV of OVX, E₂-treated female mice housed in constant conditions and killed at different times throughout the circadian day. Kiss1-containing neurons were visualized with Vector Red substrate, and c-fos mRNA was marked by the presence of silver grains. *White arrows* denote example Kiss1 cells lacking c-fos; *yellow arrows* denote example Kiss1 cells coexpressing c-fos. (b) Mean (±SEM) percentage of Kiss1 mRNA-containing neurons in the AVPV that coexpress c-fos in OVX, E₂-treated female mice killed at one of eight times throughout the circadian day. There was a significant effect of time ($P < 0.01$) with increased coexpression of Kiss1 and c-fos in the late afternoon/early evening. Values with different letters differ significantly from each other. $n = 4-6$ animals per group. From Robertson JL, Clifton DK, de la Iglesia HO, Steiner RA, Kauffman AS. Circadian regulation of Kiss1 neurons: implications for timing the preovulatory gonadotropin-releasing hormone/luteinizing hormone surge. *Endocrinology*. 2009;150 (8):3664–71. Reprinted with permission from The Endocrine Society

Fig. 18.3.

Kisspeptin-ir cells in the hamster AVPV receive SCN-derived fiber contacts expressing AVP-ir. (a) Low-power photomicrographs of AVP-ir in the AVPV, in which kisspeptin cell bodies receive extensive AVP-ir fiber contacts. (b) High-power photomicrograph showing several presumptive AVP-ir terminal boutons on a kisspeptin-ir cell body at the light level. (c) Confocal image (0.5 μ m scan taken at $\times 400$) confirming AVP-ir contacts upon kisspeptin-ir cell body and processes. In (b, c), arrows are indicative of close contacts. (d–f) Kisspeptin cells in the AVPV express the V1a receptor. (d) Low-power photomicrographs of kisspeptin-ir cells in the AVPV, (e) V1a-ir cells in the AVPV, and (f) the merged image showing overlap between kisspeptin-ir and V1a-ir. From Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, Kriegsfeld LJ. Circadian control of kiss-peptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology*. 2011;152(2):595–606. Epub 2010/12/31. Reprinted with permission from The Endocrine Society

Fig. 18.4.

Ipsilateral SCN projections gate the daily activation of GnRH cells and the AVPV Kiss1 expression rhythms in OVX + E₂ rats. (*Left*) Schematic coronal section showing the lesion location at two rostrocaudal levels from an animal that received a successful unilateral SCN lesion (*shaded*). MPO, SCN, and optic chiasm (OX) are indicated. (*Right*) Kiss1 expression is significantly lower on the ipsilateral than on the contralateral side of the lesion in animals with successful unilateral SCN lesions but not in animals in which the unilateral lesion missed the SCN. Bars, mean $\pm$ SEM. *Asterisk*, statistically different ($n = 5$ successful lesions, paired Student t test, $P = 0.016$; $n = 7$ missed lesions, paired Student t test, $P = 0.33$). From Horvath TL, Cela V, van der Beek EM. Gender-specific apposition between vasoactive intestinal peptide-containing axons and gonadotrophin-releasing hormone-producing neurons in the rat. *Brain Res.* 1998;795(1–2):277–281, with permission from Elsevier Limited. And from Smarr BL, Morris E, de la Iglesia HO. The Dorsomedial Suprachiasmatic Nucleus Times Circadian Expression of Kiss1 and the Luteinizing Hormone Surge. *Endocrinology.* 2012. Epub 2012/03/29, with permission from The Endocrine Society

Fig. 18.5.

Circadian Kiss1 expression is coupled to an oscillator within the dorsomedial SCN. **(a)** Kiss1 expression on misaligned days 1 h before dmSCN-associated activity onset, but not 1 h before the vlSCN-associated locomotor activity onset, is similar to that in aligned animals before the locomotor activity onset [one way ANOVA ($P = 0.05$)]. Bars, mean $\pm$ SEM; groups not sharing the same capital letter are statistically significant according to Tukey post hoc comparisons ($P = 0.05$). *Asterisk*, significantly different from aligned and misaligned taken together, planned comparison, $P = 0.02$. **(b)** Representative Kiss1 ISH autoradiographic films from animals killed on aligned days just before locomotor activity onset and on misaligned days just before dmSCN-associated activity onset (dm) or just before the vlSCN-associated activity onset (vl). From Smarr BL, Morris E, de la Iglesia HO. The Dorsomedial Suprachiasmatic Nucleus Times Circadian Expression of Kiss1 and the Luteinizing Hormone Surge. *Endocrinology*. 2012. Epub 2012/03/29. Reprinted with permission from The Endocrine Society

Fig. 18.6.

The activation of GnRH after kisspeptin administration is time dependent. The percentage of GnRH-ir cells expressing FOS after vehicle, 2 nmol, or 4 nmol kisspeptin administration at zeitgeber time (ZT) 1 or ZT 11 in OVX hamsters. Mean ($\pm$ SEM) percentage of POA GnRH-ir cells expressing FOS after kisspeptin administration at ZT 1 or ZT 11. From Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, Kriegsfeld LJ. Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology*. 2011;152(2):595–606. Epub 2010/12/31. Reprinted with permission from The Endocrine Society

Fig. 18.7.

SCN fibers project to GnIH-ir cells in the DMH. (a) Example injection site from an injection of biotinylated dextran amine (BDA) that filled the ventrolateral aspect of the SCN; (b) low-power photomicrograph indicating terminal fibers from the SCN project to the DMH, principally ipsilaterally. (c, d) Examples of SCN projections in close apposition to GnIH-ir cells in the DMH at the light level ((c), low power; (d), high power). (e-h) Lateralization of GnRH and GnIH activation in split hamsters. The pattern of SCN activation is lateralized in animals exhibiting two daily bouts of activity. GnIH cellular activity is lateralized and opposite to that of GnRH, indicating SCN-mediated removal of GnIH inhibition at the time of the LH surge. (e) Actogram of wheel-running activity in estradiol-implanted, OVX hamsters kept in constant light conditions (LL) from Day 1 onwards (days indicated by y axis, hours by x axis). Split hamsters were killed (*asterisk*) 1 h before the onset of one of the two activity bouts. (g) Photomicrograph of FOS activation in GnIH cells of split hamsters, showing ipsilaterally reduced GnIH activation when SCN and GnRH activation are high. Mean ($\pm$ SEM) percentage of FOS-ir (f) GnRH and (h) GnIH cells in split hamsters. *Asterisk*, significantly different from the opposite hemisphere's activational state, $P < 0.05$. From Gibson EM, Humber SA, Jain S, Williams WP, 3rd, Zhao S, Bentley GE, et al. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. *Endocrinology*. 2008;149(10):4958–69. Epub 2008/06/21. Reprinted with permission from The Endocrine Society

Fig. 18.8.

Proposed model of circadian initiation of the preovulatory LH surge in spontaneously ovulating rodents by major positive and negative regulators of GnRH neuronal activity. Kisspeptin cells in the AVPV are active at the time of the LH surge. Whereas estrogen-responsive kisspeptin cells have not been definitively shown to project specifically to GnRH neurons, the emergence and sexual dimorphism of kisspeptin cells and fibers that project to GnRH cell bodies provide compelling evidence for the direct connection between these two neural phenotypes. Connections between the GnIH and GnRH systems indicate a putative role for GnIH in modulating the negative-feedback effects of estrogen with SCN communication allowing for removal of negative feedback on the reproductive axis during the time of the LH surge. Kisspeptin cells in the ARC likely serve to modify GnRH output at the level of the terminal. See text for additional details. Figure as originally published in Williams WP 3rd, Kriegsfeld LJ. Circadian control of neuroendocrine circuits regulating female reproductive function. *Front Endocrinol (Lausanne)*. 2012;3: 60. Epub 2012 May 21 doi: 10.3389/fendo.2012.00060