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Circadian Clocks in the Ovary

Michael T. Sellix and **Michael Menaker***
Department of Biology, University of Virginia, Charlottesville VA, 22904

Abstract

Clock gene expression has been observed in tissues of the hypothalamic-pituitary-gonadal (HPG) axis. While the contribution of hypothalamic oscillators to the timing of reproductive biology is well known, the role of peripheral oscillators like those in the ovary is less clear. Circadian clocks in the ovary may play a role in the timing of ovulation. Disruption of the clock in ovarian cells or desynchrony between ovarian clocks and circadian oscillators elsewhere in the body may contribute to the onset and progression of various reproductive pathologies. Here we review evidence for clock function in the ovary across multiple species and offer a novel perspective on the role of this clock in normal ovarian physiology and in diseases that negatively impact fertility.

Keywords

rat; mouse; ovulation; rhythm; clock gene; reproduction

The Molecular Clock and Reproductive Physiology

Over the past 20 years, the field of circadian biology has come of age, primarily as a consequence of two critical developments: unraveling much of the molecular machinery that generates the near 24h oscillations and the clear demonstration that clocks exist in cells throughout the body. By now we have a good idea of how central clocks in the brain are synchronized to the environment; however, we know almost nothing about the mechanisms through which signals from the brain synchronize the clocks that exist in most peripheral organs or how peripheral clocks contribute to the functions of the organs in which they reside. It is likely that such contributions will be highly organ-specific. In all probability, elucidating the relationships between circadian rhythmicity and organ function will lead to important new insights into the physiology and pathology of specific organ systems. While it has been known for some time that many aspects of reproductive function are strongly circadian, autonomous rhythmicity in reproductive structures has only recently been explored. Though the majority of evidence from mammals suggests that some male reproductive structures (e.g. testis) do not contain cell autonomous circadian clocks [1,2], circadian clocks are present in accessory structures such as the extra-testicular ducts [3]. Circadian clocks are also present in the cells of the ovary [4–7], uterus [8–10] and oviduct [11].

The fundamental basis for circadian rhythms of physiology is a molecular clock consisting of interlocked transcriptional/translational feedback loops (for review see [12,13]). The

*Corresponding Author: PO Box 400328, University of Virginia, Charlottesville, VA 22904, mm7e@virginia.edu, Phone: 434-982-5767, Fax: 434-982-5626.

transcription factors CLOCK (or its paralog neuronal Per-Arnt-Sim (PAS) domain containing protein; NPAS2) and BMAL1 (brain and muscle arnt-like protein 1) heterodimerize and promote rhythmic transcription of the period (*per1, per2*) and cryptochrome (*cry1, cry2*) gene families. Once translated in the cytoplasm, PER and CRY proteins heterodimerize and are phosphorylated by the casein kinases (CK1ε,δ). The modified PER:CRY complexes translocate to the nucleus and repress the activity of the CLOCK:BMAL1 complex. Over several hours, PER:CRY complexes are degraded and eventually the CLOCK:BMAL1 complex is released from feedback inhibition. Though the precise kinetics of each reaction are poorly understood, it is clear that the entire process requires approximately 24h to complete, thus defining the period of the oscillator. While the period of the oscillator is relatively constant around a mean of 24h regardless of tissue, the phase of peak clock gene expression varies greatly in a cell- and tissue- dependent fashion. For instance, the rhythm of clock gene expression in the lung peaks during the early night, while that of the liver generally peaks around midnight [14]. The circadian clock gene products are basic helix-loop-helix transcription factors and as such can drive rhythmic expression of "clock-controlled genes" (CCGs) through binding at E-box promoter sequences. Recent evidence points to a multitude of genes involved in cell signaling pathways, cellular metabolism and cell cycle regulation as putative CCGs [15–17]. The cell-type specific nature of the oscillations and downstream CCG expression is likely to be critical for normal physiological function in the various components of the system.

A majority of central and peripheral tissues contain coupled cellular oscillators [18,19], which influence both endocrine and neuroendocrine processes [20–23]. For example, the neural oscillator in the suprachiasmatic nucleus of the hypothalamus (SCN) plays a well established role in the timing of the preovulatory luteinizing hormone (LH) surge [22,24]. However, normal function appears to rely on an integrated "circadian system" that synchronizes a multitude of autonomous central and peripheral clocks [19].

In the last decade, the development of rat and mouse transgenic reporter lines [14,25–28] has enhanced our ability to study the role of cell autonomous circadian clocks in mammalian physiology. To maintain physiological "rheostasis" (a term Mrosovsky has introduced to describe regulation with rhythmic steady states as opposed to the constant steady states of homeostatically regulated systems [29]), the circadian system requires adaptive "phase-synchrony" between the numerous tissue- and cell- specific circadian oscillators.

To better understand how this concept applies to the hypothalamic-pituitary-gonadal (HPG) axis, experiments are needed to elucidate the clocks' role in the physiology of each organ and cell type. Here we describe evidence for the existence of circadian clocks in the ovaries of invertebrates, non-mammalian vertebrates and mammals with an emphasis on birds and rodents. We describe the cellular distribution and functional characteristics of the ovarian clock and review the evidence for the role of this oscillator in the timing of ovarian physiology. Finally, we discuss the potential relevance of this work to the study of diseases that adversely affect female fertility. Although this review focuses on the function of the clock in the ovary, it should be clear that this tissue is but one major component of the HPG axis. For a broader discussion of the role of the circadian clock in mammalian reproductive biology as it relates to the HPG axis and general influences on fertility and fecundity, we refer the reader to several recent reviews on the topic [30–32]. In what follows we have used "clock genes" to refer to any of the genes that have been shown to participate in the feedback loop that generates basic circadian oscillations.

The Circadian Clock in the Ovary: Form and Function

Invertebrates

Clock gene expression has been observed in the ovaries of the silkworm (Bombyx mori; [33]), prawn (Macrobrachium rosenbergii; [34]) and the fruit fly (Drosophila melanogaster; [35–39]). Although circadian rhythms and/or functional consequences of clock gene expression have not been described in the silkworm or prawn, these parameters have been described in the fruit fly. In *Drosophila* ovaries, clock genes (*period* and *timeless*) are present but not rhythmically expressed [38,39]. Mutant flies lacking functional *period* (per^{01}) or *timeless* (tim^{01}) gene expression produce fewer mature oocytes and progeny after mating with a wild-type male [38]. These effects may be due to the absence of clock genes or to inbreeding depression in per^{01} mutant flies [39]. More evidence is required before any conclusions can be made regarding the importance of the circadian clock in *Drosophila* ovaries, although it is worth noting that circadian rhythmicity is essential for proper testis function in some insects [40–42]. Given the robust nature of circadian rhythms of egg-laying in *Drosophila*, it is surprising that the ovary lacks a circadian clock [43].

Non-Mammalian Vertebrates

Examination of the oviposition-ovulation cycle in domestic hens (*Gallus domesticus* [44,45] and Japanese quail (*Coturnix Japonica*; [46–48]) suggests a multi-oscillatory system that includes a central clock driving circadian rhythms of body temperature and an “ovulatory” clock responsible for the timing of oviposition [48]. In domestic hens and quail, the rhythm of ovulation-oviposition is entrained to the daily L:D cycle such that egg-laying occurs in the early morning (chicken) or middle of the afternoon (quail; [45–47]). In both species this rhythm persists in constant light with a period near 27h [46,47]. The rhythm of ovulation-oviposition in birds depends in part on the timing of LH secretion [45–47] which in turn depends on rhythmic sensitivity of the hypothalamus to circulating progesterone (for review see [44]). However, the mechanism for temporal regulation of hypothalamic sensitivity is unknown, and evidence of hierarchical control of ovulation in birds by central and peripheral oscillators is limited [49–51].

Intriguing evidence for the contribution of a functional clock within the avian ovary comes from work by Underwood and colleagues [46]. These authors examined the rhythm of core body temperature (CBT) and oviposition in intact or ovariectomized (OVX) Japanese quail housed in either constant light (LL) or constant darkness (DD). In quail, as in many endotherms, there is a robust circadian rhythm of CBT, peaking around the middle of the subjective day [46]. The rhythm of CBT is entrained to the 12:12 L:D cycle and free-runs (persists with an endogenous period) in constant conditions. In LL at 10 and 100 lux, the circadian rhythm of CBT free-runs with a period >24h and is synchronized with the rhythm of oviposition. When placed in DD, animals cease to ovulate and the rhythm of CBT free-runs with a period closer to 22h. Surprisingly, ovariectomy abolished rhythms of CBT when birds were placed in LL. Although birds have photoreceptors in both their eyes and brains (for review see [52]), blocking visual cues with opaque cones affixed to the eyes resulted in two simultaneous rhythms of CBT, one with a period <24h and another >24h associated with the rhythm of oviposition. These data strongly suggest the influence of multiple dissociable circadian oscillators on the rhythm of CBT [46]. Perhaps one oscillator, (in the basal hypothalamus?) drives the short period rhythm and another oscillator (in the ovary?), produces the longer period rhythm associated with oviposition. While the source of the long period ovary-dependent oscillation remains unknown, one possibility is that the ovarian clock drives a long period (>24h) rhythm of steroid hormone secretion that in turn induces the observed rhythm in CBT.

Evidence for the existence of a clock in the ovary itself was recently reported in Japanese quail and domestic hens [47,53]. Expression of clock genes including *per2, per3, clock and bmal1* was detected in multiple quail tissues including the liver, lung, heart, kidney, spleen and ovary [53]. Furthermore, strong diurnal rhythms of quail *per2 (qPer2)* and *qPer3* mRNA expression in the granulosa and thecal cells of fully mature F1 follicles were observed [47]. Rhythms of *qPer2* and a tendency toward cycling in *qPer3*, *clock* and *bmal1* were also detected in F1 follicles from quail housed in constant light. Diurnal rhythms of the cholesterol transporter steroidogenic acute regulatory protein (*StAR*) and the steroid biosynthetic enzyme 3β-hydroxysteroid dehydrogenase (*3-βhsd*) were observed in F1 follicles [47]. Analysis of the promoter region of *StAR* in transiently transfected primary cultures of chick granulose cells revealed transcriptional activation of *StAR* gene expression by the CLOCK:BMAL1 heterodimer [47]. It seems likely that the ovary in quail (and domestic hens) contains a circadian clock, specifically within granulosa and thecal cells, which plays a role in the timing of steroidogenesis. As Ball suggests [48], this supports the novel hypothesis that the ovarian clock in birds may contribute to the circadian rhythm of ovulation by driving rhythmic positive steroid hormone feedback on the hypothalamus. The gross similarities among rhythms of ovulation-oviposition in birds, ovulation during the estrous cycle in rodents and the menstrual cycle in humans (for review see [44,45] and Box 1) suggest that the circadian clock in cells of the mammalian follicle could play a similar role in ovarian physiology.

Box 1

Comparative physiology of preovulatory LH secretion and the timing of ovulation

Circadian rhythms of ovulation in birds and rodents are linked to the timing of LH secretion, itself driven by circadian pacemakers in the SCN (for review see [44,45]). Although control of the LH surge by the circadian clock in the SCN has been demonstrated in rodents (e.g. rats, mice and hamsters), a similar role for this structure remains unclear in women. However, diurnal rhythms of LH secretion during the menstrual cycle, which correlate strongly with the circadian rhythm of serum cortisol and the light:dark cycle, have been repeatedly observed in female subjects [93–95]. Further, it has been reported that ovulation occurs within 5–15 hours of peak LH secretion, further supporting the notion that the timing of ovulation in women may be linked to the circadian system [94]. Future experiments may provide definitive evidence linking the activity of pacemaker neurons in the SCN with the timing of ovarian physiology in women. Unraveling these relationships may prove critical to understanding the role of circadian clock function in diseases that negatively impact fertility.

Mammals

Clock gene expression has been described in the ovaries of rats [4–7,54], mice [31,55] and ruminants (ovine, bovine; [56]). *In situ* methods were used to localize clock gene expression to the granulosa (GC), thecal (TC) and luteal (LC) cells of the rat ovary [4,5]. Analyses with quantitative real-time (qPCR) indicated that *per1* and *per2* mRNA expression peaked in the early night, was rhythmic regardless of estrous cycle phase and persisted when rats were placed in constant darkness [5]. Expression of *per1* and *per2* mRNA was also observed in interstitial glandular tissue, corpora lutea, pre-antral, antral and pre-ovulatory follicles. Furthermore, high-power confocal microscopy measured time-dependent shuttling of PER1 and PER2 proteins between the cytoplasm and nucleus in luteinized granulosa and thecal cells [5]. Within the same year circadian rhythms of *per2* and *bmal1* mRNA were reported in rat GCs and TCs [4]. Further, real-time PCR revealed a significant increase in *bmal1* expression at ZT18 (ZT12=lights off in a 12:12 L:D cycle) on proestrus, suggesting an effect of the preovulatory

LH surge, which occurs between ZT12-18, on the ovarian clock [4]. To explore this, the authors treated hypophysectomized immature female rats with a priming dose of equine chorionic gonadotrophin (eCG) followed by treatment with human chorionic gonadotrophin (hCG). Treatment with eCG alone, which induces development of immature follicles, failed to produce a significant increase or robust diurnal rhythm of *per2* or *bmal1* expression. However, subsequent treatment with hCG, which acts at LH/CG receptors and is known to induce follicular rupture, produced a robust diurnal rhythm of clock gene expression in the rat ovary [4]. These data suggest that the rat ovary contains gonadotrophin-sensitive circadian oscillators.

In vitro evidence for a cell autonomous circadian clock in specific cell types within the ovary was first reported by He and colleagues [6,54]. They monitored real-time bioluminescence in dexamethasone (DEX) synchronized primary cultures of GCs and LCs from *per2-luciferase* (*per2-luc*) transgenic rats. A circadian rhythm of *per2-luc* expression was observed in LCs from pregnant rats or immature rats primed with eCG and hCG, whereas naïve GCs failed to show persistent high amplitude rhythms [54]. In a subsequent study, these authors examined the response of primary GC cultures to gonadotrophin treatment [6], but did not detect rhythms of *per1* mRNA expression in GCs from immature follicles. However, *per1* mRNA was rhythmic in LCs from pubertal rats and could be stimulated by gonadotrophins through CRE-mediated transactivation [6]. These data indicate that follicular cells are able to express cell autonomous circadian rhythmicity, and that while these oscillators can be affected by gonadotrophins, the effect depends on the differentiated state of the cell (e.g. GC vs. LC).

In a recent study our laboratory examined the response of the ovarian circadian clock of rats to phase-shifts of the 12:12 L:D cycle and found that entrainment requires humoral, but not neural, inputs [7]. Further, circadian rhythms of *per1-luciferase* gene expression were measured in individual follicular cells taken from gonadotrophin-primed immature rats and maintained as mixed granulosa/thecal cell monolayer cultures (Figure 1a). In addition, phase-dependent responses to LH or FSH treatment were observed [7]. The stimulatory effects of gonadotrophins on the circadian clock in cultured granulosa cells agree with results reported by others [6]. Thus, follicular cells may normally be entrained to the environment by humoral cues, most likely pituitary gonadotrophins, acting in a phase-dependent manner. However, these data do not provide evidence for a functional link between the clocks in the ovary and ovarian physiology. To address this, it will be necessary to examine the effects on ovarian physiology of disrupting or eliminating the circadian clock. Though complicated by significant caveats, evidence suggests that global clock gene mutations (e.g. $clock^{\Delta 19}$ mutant, $bmal1^{-/-}$ or *per1/2* double KOs) fail to alter follicular growth and morphology (see [30,57–59] and Box 2). The use of tissue and cell specific targeted genetic deletion of clock gene expression may substantially improve our understanding of how individual circadian oscillators within the HPG axis contribute to reproductive physiology.

Box 2

The effects of clock gene mutations on reproductive physiology

Several recent studies describe the effects of disrupting circadian clock function on mammalian reproductive physiology [10,30,57,59,96,97]. Reduced fertility and fecundity have been reported in middle-aged, but not young, $per1^{-/-}$ and $per2^{-/-}$mice [98]. Examination of dominant negative $clock^{\Delta 19}$ mutant mice reveals prolonged estrous cycles with extended periods of estrus and a reduced amplitude or complete absence of a proestrous LH surge [10,57]. These mice have morphologically normal ovaries, regularly mate and produce fertilizable ova, though a significant proportion of embryos and full-term fetuses are reabsorbed [57]. Evidence suggests that irregular estrous cycles in *clock* mutant mice are due to abnormal rhythms of vasopressin (AVP) expression in SCN neurons and reduced AVP1a receptor in SCN target regions in the hypothalamus [99].

Several investigators have observed significantly impaired fertility and fecundity in *bmal1*$^{-/-}$ mice [58,59,80]. *Bmal1*$^{-/-}$ mice display decreased steroidogenesis due to reduced expression of steroidogenic acute regulatory protein (a rate limiting enzyme in steroid hormone synthesis [80]). Although *bmal1*$^{-/-}$ mice exhibit abnormally long estrous cycles, they produce viable and fertilizable ova [58]. Compared with wild-type littermates, these animals display a higher incidence of implantation failure due to reduced progesterone secretion from the corpus luteum [58]. It is worth noting that *StAR* and *bmal1* are apparently arrhythmic in corpora lutea, suggesting that regulation of steroidogenesis by *bmal1* occurs through a non-clock associated pathway.

An important caveat to these results is worth noting. Most of the studies examining the effects of clock gene mutations on fertility have been conducted with mice housed in 12:12 L:D cycles. Thus, these experiments fail to account for the impact of photic masking, which may override the effects of genetic mutations. A recent report examining the effects of the *clock*$^{\Delta 19}$ mutation in mice housed in constant dark revealed a significant increase in abnormal cycling and pregnancy losses [10]. Further, scrutiny reveals a trend towards a reduced number of oocytes and fertilized embryos in the oviducts from *bmal1*$^{-/-}$ mice on the third day after mating with a wild-type male [58]. Thus, while the preponderance of evidence suggests that follicular maturation and ovarian morphology remain normal in both *clock* and *bmal1* mutant mice held in L:D cycles, it is possible that the sensitivity of the ovarian follicle to gonadotrophins is impaired by the circadian deficits.

To investigate a potential physiological role for the clock in the rat ovary, we suppressed endogenous LH secretion with a selective gonadotrophin releasing hormone (GnRH) receptor antagonist (Cetrorelix; CET) and examined the phasic response of the ovary (i.e. ovulation) to exogenous gonadotrophin treatment (Figure 1b, 1c [60]). Animals kept in a 12:12 L:D cycle that were treated with CET on the afternoon of diestrus or the early morning of proestrus displayed a robust diurnal rhythm of ovulation in response to exogenous LH such that animals ovulated more often when injected during the dark phase on both days (Figure 1b). A repeat of this experiment with rats kept in constant dim light revealed an equally robust circadian rhythm of ovulation with a peak during the subjective night (Figure 1c). These data imply that the circadian responsiveness of the ovary, independent of the timing of endogenous gonadotrophin secretion, contributes to the timing of ovulation by setting a window of sensitivity to LH. Additional experiments are needed to discriminate between the role of clocks in the ovary itself and/or the possible contribution of other endocrine or neural inputs to this rhythm of ovarian sensitivity.

A Paradigm Shift: How the Circadian Clock in the Ovary May Contribute to the Timing of Ovulation

Since the elegant work of Everett and Sawyer, it has been clear that a neural timing system drives the rhythmic secretion of LH and subsequent ovulation [61,62]; see Figure 2a). However, it was approximately 22 years before the central circadian clock was found to be localized in the SCN [63]. Since then, lesions of the SCN have been shown to disrupt the timing of LH secretion, ovulation and normal cycling in female rats [64,65]. These and many other reports strongly support the classical paradigm for the timing of ovulation in rodents ([24]; Figure 2a). That is, the circadian oscillator in the SCN drives rhythmic patterns of GnRH release, which in turn stimulates LH secretion on the afternoon of proestrus. This preovulatory surge of LH initiates a cascade of events leading to follicular rupture and oocyte release [66,67]. However, recent studies suggest that the GnRH neuron may also be an autonomous circadian oscillator [68–71]. Rhythms of clock gene expression have been observed in cultured immortalized GnRH neurons [68,69] and in mouse GnRH neurons *in vivo* [70]. There is even limited evidence

supporting circadian clock gene expression in pituitary cells [72–76]. These data, together with the evidence presented above for the participation of the ovary's own clock in the control of ovulation, support a novel paradigm: rather than responding to a linear hierarchical system, events like ovulation which are controlled by the HPG axis are timed by a multi-oscillatory system that depends on synchronization among its coupled components (Figure 2b).

To understand the role of the ovarian clock in the timing of ovulation, it will be necessary to uncover the links between the clock, clock-controlled gene expression and the molecular processes associated with follicular rupture. The proestrous LH surge initiates a complex and generally well understood cascade of molecular events resulting in weakening and eventual rupture of the follicular wall [66,67]. The response has been described as an "inflammatory" reaction involving the activation and secretion of enzymes such as cyclooxygenase-2, matrix metalloproteinases and prostaglandins from the cells lining the follicular wall [66,67]. Thus, one possible link between the circadian clock in the ovary and the timing of ovulation could involve an increase in prostanoid signaling [66,67]. Prostaglandin E2 and prostaglandin F2α are involved in the inflammatory response that precedes rupture of the follicular lumen [77]. The activity of the enzyme cyclooxygenase-2 (*cox2*) is the rate limiting step in prostaglandin (PG) synthesis. It was recently reported that *cox2* transcription is partially regulated through E-box DNA binding sequences located in its promoter region that are known targets of the CLOCK:BMAL1 transactivator complex [78,79]. The timing of *cox2* expression relative to ovulation is highly conserved, such that *cox2* mRNA begins to increase approximately 10h before ovulation in several species [77]. While the presence of an E-box consensus sequence in the *cox2* promoter does not prove that *cox2* transcription is regulated by the core clock, E-box mediated transcription is required for activation of *cox2* expression by gonadotrophins [78]. In light of these data, we propose a working model (Figure 3) in which CLOCK:BMAL1 heterodimers bind to and activate clock controlled transcription of *cox2* mRNA on the day of ovulation. An increase in COX2 and in turn PG synthesis, in anticipation of the LH surge, would provide a window for follicular rupture induced by the surge. In addition to the process of follicular rupture, recent evidence suggests circadian clock control of steroidogenesis, a facet of ovarian physiology critical for reproductive success [58,80]. The timing of follicular rupture and steroidogenesis may both be regulated by the circadian clock in the ovary and act in concert to facilitate the timing of ovulation.

Circadian Clocks in the HPG and Disorders of the Female Reproductive System: A Working Hypothesis

Disruption of the circadian system in the HPG may be a causative factor in diseases affecting fertility. We know that conditions resulting in disrupted coordination or synchronization within and between circadian clocks in the brain and periphery can have significant negative impacts on health. For example, transient phase shifts associated with transmeridian travel and rotating shift work activate the stress axis, and increase cancer risk, sleep/mood disorders and the incidence of digestive disorders [81–84]. It has been suggested that these effects are directly linked to desynchronization within and between circadian clocks in the brain and periphery [85]. These conditions are also known to affect reproductive physiology and fertility in humans, including but not limited to altered menstrual cycles, increased menstrual pain, altered follicular phase length, changes in the level of FSH secretion, low birth weights and greater incidence of spontaneous abortion [32,86]. Further, a recent genetic screen in Finland revealed that a polymorphism of the clock gene *arntl (bmal1; arntl TT)* increased the percentage of miscarriages [87]. Normal reproductive physiology may well depend on coordination within and between circadian clocks in the HPG axis (Figure 4a). Diseases like polycystic ovarian syndrome (PCOS), endometriosis or various malignant endocrine tumors which are common and have adverse effects on fertility, could be caused or exacerbated by desynchronization between circadian clocks in the ovary, pituitary, uterus, oviduct and mediobasal hypothalamus

([32,88,89]; Figure 4b). As an example, the etiology of PCOS may be indirectly linked to the timing of the circadian clock. While considerable debate surrounds the exact etiology of the disease, it is generally believed that excessive androgen secretion during development is an underlying factor [90]. Recent evidence suggests an interaction between androgen receptor stimulation and the timing of circadian rhythms of behavior, itself dependent on rhythms of clock gene expression in SCN neurons [91,92]. Persistent stimulation by circulating androgens during development may alter the phase or period of the clock in central and peripheral tissues, resulting in potentially harmful desynchrony among constituent oscillators of the HPG axis (Figure 4b). A shift away from the classic "top-down" view of the timing of reproductive physiology should inspire researchers to examine the effects of perturbing tissue specific clocks through global, targeted or conditional genetic ablation on the etiology of complex disorders of the reproductive system.

Concluding remarks

We have highlighted current evidence for the existence of circadian clocks in the ovaries of several species. In addition, we have summarized what little is known regarding the physiological function of ovarian clocks. We propose a revised paradigm in which circadian clocks at each level of the HPG axis contribute to the timing of reproductive events, with specific emphasis on the possible role of the ovary's circadian clock in the timing of ovulation. Finally, we hypothesize that synchronization within and between these clocks contributes to rheostasis in the reproductive system whereas disruption of this synchrony may exacerbate, or in some cases even cause, disease that adversely affects fertility.

Glossary

Suprachiasmatic nucleus (SCN)	Central circadian clock located in the mediobasal hypothalamus just above the optic chiasm
Luteinizing hormone (LH)	Gonadotrophin released from the anterior pituitary gland by stimulation from gonadotrophin releasing hormone (GnRH) from the hypothalamus. The surge of LH on proestrus stimulates follicular rupture
Follicle stimulating hormone (FSH)	Gonadotrophin released from the anterior pituitary gland by stimulation from gonadotrophin releasing hormone (GnRH). FSH stimulates growth and maturation of the ovarian follicle
Zeitgeber time (ZT)	Time scale relative to exogenous light:dark cycle: ZT0 = light on and ZT12 = lights off
Circadian time (CT)	Subjective time scale dependent on the animal's activity: CT0 = activity end and CT12 = activity onset
Granulosa cell (GC)	Cells that surround the oocyte and follicular antrum in multiple layers. Granulosa cells are physically separated from thecal cells by the basal lamina. Granulosa cells synthesize and secrete estradiol and are luteinized following stimulation by the LH surge on proestrus
Thecal cell (TC)	Cells located in the theca interna between the innermost granulosa cells and the non-hormone secreting epithelial cells located in the theca externa. They normally produce progesterone that is primarily converted to androgens, secrete estrogen and are luteinized following stimulation by the LH surge on proestrus

Luteal cell (LC)	Progesterone-secreting cells of the corpus luteum

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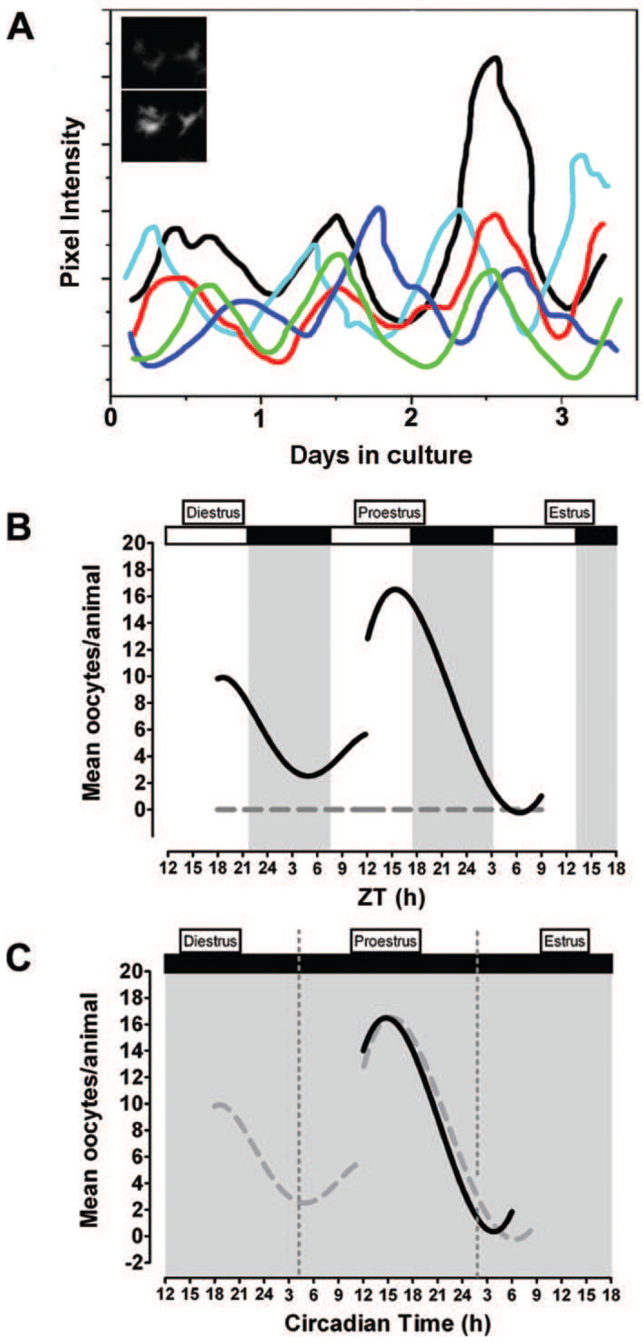

Figure 1.

A circadian clock in the rat ovary may contribute to the timing of ovulation. (a) Schematic representation showing circadian rhythms of *period1-luciferase* gene expression in individual granulosa/thecal cells. Inset graph: Images of trough (top panel) and peak (bottom panel) *per1-luciferase* gene expression in a representative granulosa/thecal cell recorded with an intensified CCD camera. Based on data from [7]. Schematic representation of (b) diurnal and (c) circadian rhythms of ovulation in response to exogenous LH in the absence of endogenous LH secretion. Animals housed under (b) a 12:12 L:D cycle or (c) constant dim light were injected with the GnRH receptor antagonist Cetrorelix on diestrus or proestrus to suppress endogenous LH secretion followed by timed injections of equine LH (solid black lines). LH-treatment during

the subjective night on both diestrus and proestrus (L:D) or proestrus alone resulted in more frequent ovulation and significantly more oocytes/ovulation. Animals treated with sterile saline (gray dashed line in (b)) failed to ovulate regardless of injection time. The open and solid bars at the top of the figure indicate the light and dark portions of the L:D cycle. The solid gray background in (c) indicates that animals were maintained under constant dim light. Dashed gray lines in (f) are data from (b) re-plotted to emphasize the similarity of the results. Panels (b, c) modified from [60].

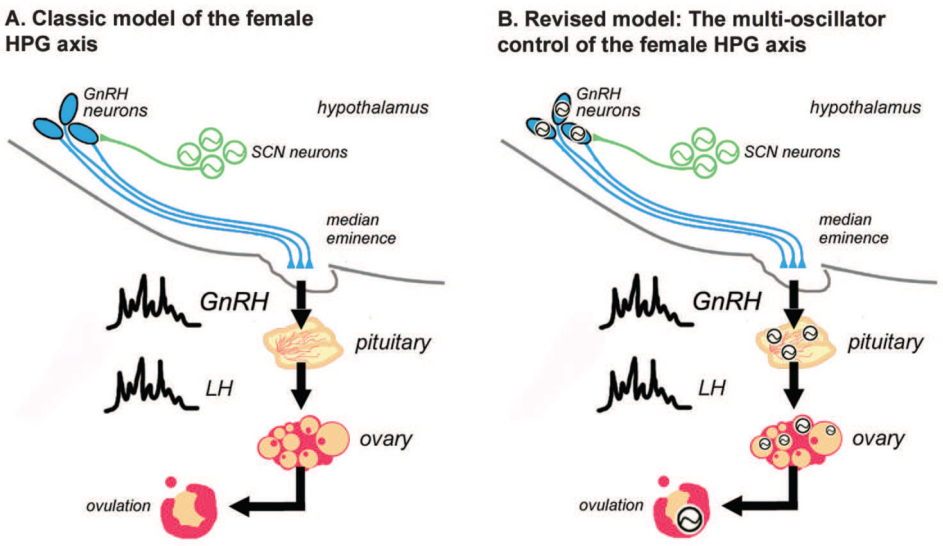

Figure 2.

A paradigm shift: How the multi-oscillator HPG axis controls the timing of reproductive physiology. (a) Classic model for hypothalamic-pituitary-gonadal (HPG) axis regulation of mammalian reproductive physiology. On the afternoon of proestrus, the circadian clock in the SCN drives rhythmic release of GnRH that stimulates rhythmic secretion of LH. Circulating LH then stimulates the ovarian follicle leading to rupture and oocyte release. According to the classic model, the timing of events in this system is dependent solely on timing cues from the pacemaker neurons in the SCN. (b) A revised model for the "multi-oscillator circadian system" in the HPG axis emphasizing the existence of circadian oscillators in each component of the axis. Synchronization between SCN pacemakers, GnRH neurons, pituitary cells and ovarian cells is necessary for proper timing of physiological events controlled by the HPG. Based on this model, we hypothesize that disrupting circadian phase relationships among these tissues will have negative effects on reproductive physiology. Green circles containing sine waves represent SCN pacemaker neurons. Black circles containing sine waves represent rhythmic circadian clock gene expression within the cells of each tissue or region. Potential feedback relationships among the oscillators have been omitted for clarity. Gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH), Suprachiasmatic nucleus (SCN).

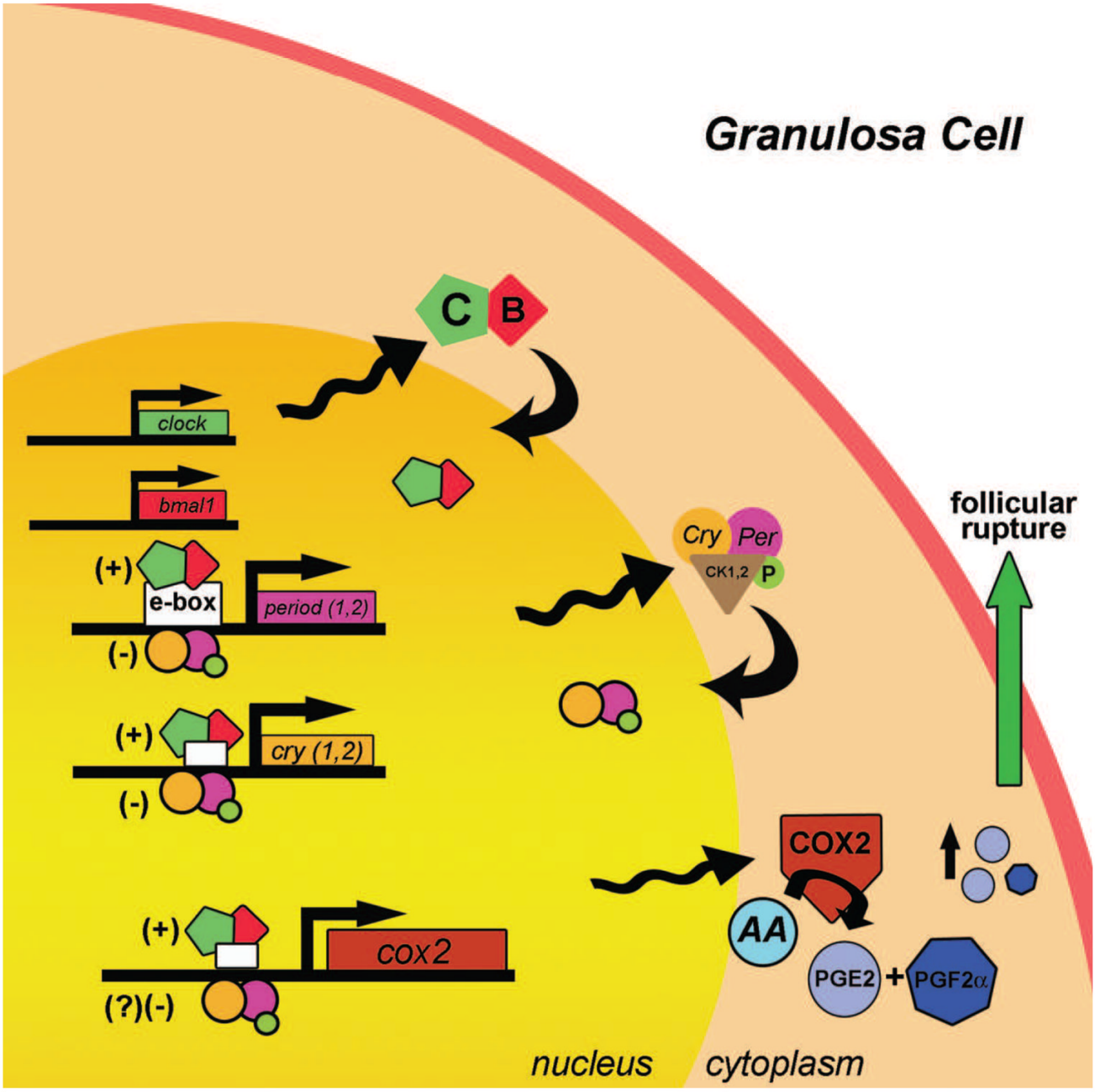

Figure 3.

The molecular clock in mammalian follicular cells may drive the expression of clock-controlled genes necessary for ovulation. Circadian clock genes, including activators [BMAL1 (B); CLOCK (C)] and repressors [*period (per)* and *cryptochrome (cry)*], are rhythmically expressed and phosphorylated by casein kinases in granulosa cells. *Cyclooxygenase-2 (cox2)*, the rate limiting enzyme for prostanoid synthesis, has E-box sequences in its promoter region, and evidence suggests that CLOCK:BMAL1 heterodimers can bind to and activate *cox2* transcription [78,79]. Circadian rhythms of *cox2* mRNA expression may result in rhythmic accumulation of COX2 enzyme. In turn, rhythms of COX2 enzyme expression may lead to rhythmic synthesis and accumulation of PGE2 and PGF2α. Increased levels of prostanoid

synthesis, particularly in response to a surge in LH secretion, are associated with follicular rupture. Thus, circadian rhythms of *cox2* mRNA synthesis might indirectly contribute to the timing of ovulation by establishing a ready pool of LH-inducible prostaglandins. Transactivation by BMAL1:CLOCK is indicated by (+); repression of BMAL1:CLOCK activity by PER:CRY is indicated by (−). Arrowheads attached to sine waves indicate rhythmic transcription/translation. Curved arrows indicate nuclear translocation. Abbreviations: arachidonic acid (AA); prostaglandin E2 (PGE2); prostaglandin F2α (PGF2α); phosphorylation (P); Casein kinase 1,2 (CK1,2).

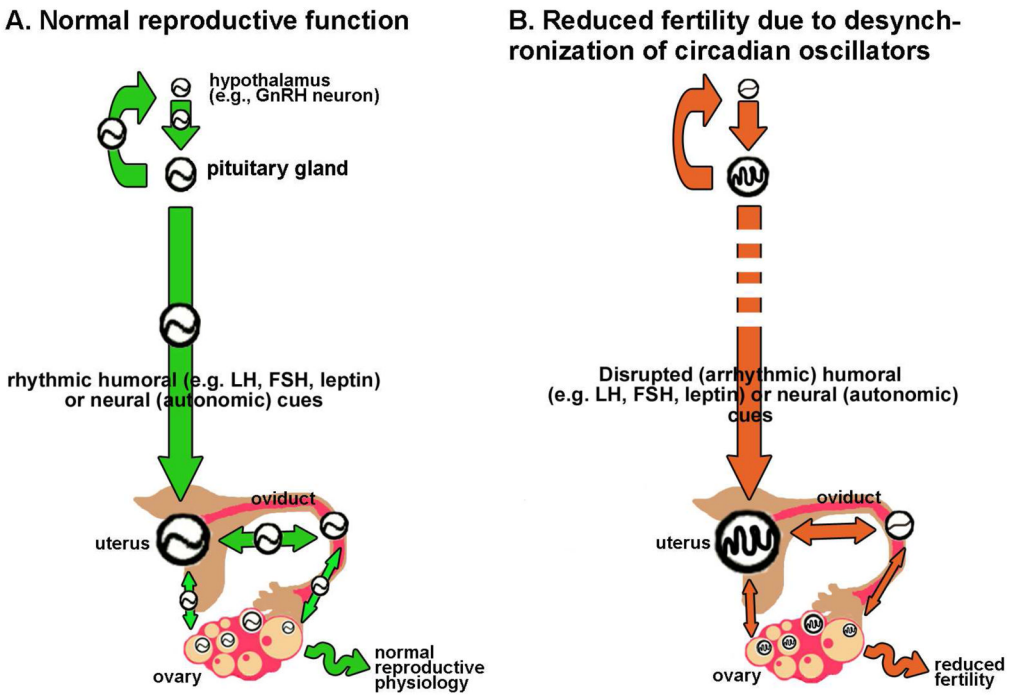

Figure 4.

A synchronized circadian system in the HPG may be necessary for rheostasis. (a) We suggest that rheostasis in the reproductive system depends on synchronization within and between circadian clocks in the HPG axis. For normal function circadian clocks in hypothalamic neuroendocrine cells (e.g. GnRH neurons), pituitary gland, uterus, oviduct and ovary (represented by circles containing a sine wave) must be appropriately synchronized. Regular temporal cues [indicated as green arrows in (a)] originating in the SCN and transduced by nervous and humoral outputs to the periphery to drive coordination of central and peripheral clocks. Temporal cues originating in peripheral oscillators (e.g. the uterus) may also alter timing in nearby peripheral oscillators. For clarity, several additional links and feedback circuits have been omitted from this schematic. (b) We hypothesize that disrupting normal synchronization, either by reducing the amplitude or robustness of circadian clocks in target tissues [indicated by circles containing abnormal waveforms] or by altering the rhythmicity or amplitude of temporal cues [indicated by red arrows] of central or peripheral origin may exacerbate (or even cause) diseases associated with reduced fertility.