Daily rhythms count for female fertility

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Practice points

- Ovulation is triggered by a marked and transitory LH surge occurring at a stage when oocytes are mature and at the end of the daily resting period.
- The LH surge is gated by high circulating estradiol produced by the ovaries and a circadian signal driven by the hypothalamic master biological clock.
- The circadian signal is forwarded by the hypothalamic master clock towards two distinct neuronal populations. The kisspeptin expressing neurons which stimulate and the (Arg)(Phe)related peptide (RFRP) expressing neurons which inhibit the GnRH-driven LH secretion.

Research agenda

- The circadian regulation of the pre-ovulatory LH surge needs to be investigated in diurnal rodents and non-rodent species
- The effect of circadian disruption (jet-lag or shift work) on reproductive capacity needs to be experimentally investigated
- Rigorous epidemiologic studies should be performed to assess the effect of shift work on fertility of women.

Abstract

Female ovulation depends on a surge in circulating luteinizing hormone (LH) which occurs at the end of the resting period and requires high circulating estradiol. This fine tuning involves both an estradiol feedback as an indicator of oocyte maturation, and the master circadian clock of the suprachiasmatic nuclei as an indicator of the time of the day. This review describes the mechanisms through which daily time cues are conveyed to reproductive hypothalamic neurons to time the pre-ovulatory surge. In female rodents, neurotransmitters released by the suprachiasmatic nuclei activate the stimulatory kisspeptin neurons and reduce the inhibitory RFRP neurons precisely at the end of the afternoon of proestrus to allow a full surge in LH secretion. From these findings, the impact of circadian disruptions (during shift or night work) on female reproductive performance and fertility should now be investigated in both animal models and humans.

Key words: female reproduction, pre-ovulatory LH surge, circadian clock, kisspeptin, RFRP, estradiol, shiftwork.

1- A dedicated hypothalamic network controls reproductive activity

Gonadal activity depends primarily on a set of neurons located in the hypothalamus and producing the gonatotropin releasing hormone (GnRH). These neurons are scattered in the preoptic area and the vascular organ of the lamina terminalis, but they mostly project at the median eminence where they release GnRH into the portal blood to further activate the synthesis and release of two gonatropins, luteinizing (LH) and folliculo-stimulating (FSH) hormones, from the pituitary gonadotrophs. In females, FSH promotes follicular growth and sex steroid production while LH triggers ovulation of the mature follicles when the circulating level of estradiol is high enough to attest follicle maturation.

Various (neuro)transmitters have been proposed to regulate GnRH neuronal activity, but in 2003, the finding that mutations in the gene encoding the receptor of kisspeptin (Kiss1R, formerly GPR54) induces idiopathic hypogonadotropic hypogonadism in humans and mice [1][2] shed light on the pivotal role of kisspeptin on the regulation of GnRH neurons. The Kiss1 gene encodes a family of peptides generated from an initial 145 amino acid kisspeptin (Kp) propeptide, Kp145, which is cleaved into peptides of different sizes from Kp54 (previously named metastin) to Kp10. The discovery of Kp and its major role in reproductive function have been a milestone in the field of reproductive biology. An increasing number of studies now indicate that Kps are critical regulators of sexual differentiation and maturation as well as of normal adult reproductive functioning across mammalian species, including humans [3]. Kp neurons are localized within two hypothalamic areas, in the arcuate nucleus (ARN) and the rostral periventricular nucleus of the third ventricle, also called anteroventral

periventricular nucleus (AVPV), or the preoptic area (depending on species). They send projections mainly to the GnRH neuron cell bodies (AVPV Kp neurons) and nerve terminals (ARN Kp neurons) [4][5][6]. Importantly, AVPV presents a marked sexual dimorphism with much more Kp neurons in females and it plays a specific role in driving the pre-ovulatory GnRH/LH surge [4][7][8]. Kiss1R is highly expressed in GnRH neurons but also in other brain areas and in endocrine tissues like the pituitary gland, ovary, and placenta [9][10][11]. Kp has a very potent stimulatory action on GnRH release and therefore gonadotropin secretion in all mammalian species investigated so far [12][13][14][15]. Central injection of doses as low as 0.1 to 1 pmole Kp10 is sufficient to evoke robust LH secretion in rodents and primates [13][15]. The essential role of the Kiss1/Kiss1R complex in the central regulation of the gonadotropic axis is attested by the profound impaired reproduction (abnormal sexual maturation, small uterus, ovaries without mature follicles, no estrous cycle) associated to mutations in Kiss1 [16][17] or Kiss1R [1][18][19] in mammals, including humans.

Other neurotransmitters and hormones have been reported to regulate GnRH neuron activity, albeit not to the same extent as Kp, such as glutamate which stimulates Gnrh gene expression and GnRH release during the LH surge [20][21] and nitric oxide (NO) which has been reported to coordinate GnRH neuronal activity [22]. Notably, recent studies indicate that another neuropeptide belonging to the same RF-amide peptide family as Kp, RFRP-3 (the mammalian homolog of avian gonadotropin inhibitory hormone (GnIH)), acts at several central sites to regulate reproductive activity. RFRP-3 is encoded by the Rfrp gene (also encoding RFRP-1 with moderate if any effect on the reproductive axis) expressed in neurons exclusively located in the dorsomedial hypothalamus [23]. RFRP neurons project to various brain areas including the preoptic area and within the vascular organ of the lamina terminalis

(OVLT) where RFRP fibers make contact with GnRH neurons and the AVPV/medial preoptic nucleus where RFRP fibers make contact with Kp neurons [23][24][25]. The main RFRP-3 binding site is reported to be the receptor GPR147 (also known as NPFF1R), but it is possible that other receptors are activated by RFRP-3 due to cross reactivity to other RF-amides receptors [26]. GPR147 is expressed in various brain areas notably those related to the central control of reproduction. Thus a significant number of GnRH neurons (15-33%) and Kp neurons (5-25%) express GPR147 [24][27]. Further, electrophysiological investigation on mouse hypothalamic sections has demonstrated a direct effect of RFRP-3 on GnRH neuronal firing rate with either inhibitory (41%) or stimulatory (12%) action [28]. Unlike Kp, RFRP-3 is mostly reported to inhibit reproductive activity [23][29][30]. However, recent studies have revealed a sex-dependant effect of the peptide. Thus in Syrian hamsters and mice, a central injection of RFRP-3 increases GnRH neuronal activity and LH secretion in males, whereas in females it reduces the amplitude of the LH surge [23][31][32]. Moreover, RFRP-3 appears to have an additional direct hypophysiotropic effect in ewes [29], although this is still disputable in rodents.

2. The critical role of the sex steroid feedback

Sex steroids produced by the gonads have long been known to feedback on the hypothalamo-pituitary axis in order to exert a retrocontrol of reproductive activity. In males, testosterone exerts a sustained negative feedback whereas in female mammals, the feedback is more complex as estradiol exerts positive or negative feedback according to the stage of the ovarian cycle and the concentration of circulating estradiol.

During the first part of the female reproductive cycle, the low level of circulating estradiol induces a negative feedback, whereas upon oocyte maturation (at the end of the follicular phase in humans or pro-estrus in rodents), higher estradiol concentration exerts a positive feedback, causing a synchronized activation of GnRH neurons leading to GnRH release in the hypophyseal portal blood and finally the pre-ovulatory LH surge [33]. The effect of estradiol is mediated via two types of nuclear estrogen receptors (ER) which induce long lasting genomic action, ER α and ER β [34][35], but it can also have a rapid action via the membrane bound estradiol receptor GPR30 [36][37].

Although GnRH neurons express ER β , they do not appear to be the direct site of the estradiol feedback. Indeed, mice bearing a GnRH neuron-selective deletion of ER β exhibit normal cycles and negative feedback [38], leaving the putative role for ER β in GnRH neuron activity still an open question [34].

In contrast to GnRH, Kp neurons have a high density of ER α and are therefore considered as the pivotal node for the estradiol feedback [7][39][40]. Interestingly, in rodents the estradiol feedback depends on Kp neuron localization as estradiol stimulates Kiss1 expression in the AVPV while it inhibits Kiss1 expression in the ARN [40][41][42][43]. In non-rodent mammals, a similar differential regulation by estradiol is also observed with a stimulatory effect in the rostral periventricular/preoptic area and an inhibitory effect in the ARN [44][45]. It has been proposed that AVPV Kiss1 activation requires an estrogen response element (ERE)-dependent pathway, whereas inhibition of Kiss1 expression in the ARN involves ERE-independent mechanisms [41][46]. Notably, mice lacking ER α specifically in Kp neurons

display an advanced puberty and extremely irregular estrous cycles, further demonstrating the role of Kp neurons in conveying the estradiol feedback towards the GnRH neurons [47].

The effect of estradiol on RFRP neurons remains questionable according to studies. Indeed a portion of RFRP neurons has been shown to co-express ER α in female rodents [23][48]. In some studies estradiol has been shown to induce c-Fos expression in RFRP neurons [23] and to stimulate *Rfrp* expression [49]. In contrast other studies have reported that estradiol decreases *Rfrp* expression [48][50] or has no effect [32][51]. Thus, additional studies are required to clarify whether estradiol regulates RFRP neurons and, if so, to assess putative sex differences in its effect.

3- The hypothalamo-pituitary-gonadal axis displays rhythms

In adulthood, the reproductive axis is highly rhythmic with different periodic time scales going from a few minutes (GnRH pulsatile release) to days (LH surge in female), weeks (ovarian cycles in female) or months (seasonal reproduction).

GnRH secretion is pulsatile and this is critical to induce proper gonadotropin secretion as there is a tight correlation between GnRH and LH pulsilities [52]. Thus, discontinuous but not constant administration of exogenous GnRH is capable of restoring reproductive activity in patients suffering from Kallmann syndrome [53]. Increasing evidence suggests that ARN Kp neurons which co-express neurokinin and dynorphin (KNDy neurons) drive GnRH

pulsatility [54][55]. Notably, Kp release in the stalk-median eminence is pulsatile [56], and pulsatile Kp drives LH secretion in juvenile monkeys [57]. A recent study reported that pulsatile administration of Kp was able to evoke a dramatic synchronous activation of GnRH gene transcription with robust stimulation of GnRH secretion in murine cultured hypothalamic explants [58].

In female mammals, ovarian activity displays regular cycles (menstrual cycles in women, estrous cycles in rodents) driven mostly by the change in circulating pituitary gonadotropins. During the first part of the reproductive cycle (follicular phase in women; metestrus-diestrus in rodents), FSH secretion increases, leading to the recruitment and development of ovarian follicles. The growing follicles cause a progressive increase in estradiol associated with a higher LH receptor expression in granulosa cells [59]. During this early phase, LH pulses occur with a high frequency and stable low amplitude. The second part of the reproductive cycle (luteal phase in women; proestrus-estrous in rodents) begins with a marked and transient secretion of LH. This LH surge induces the ovulation of mature follicles, the continuation of oocyte meiosis, and an arrest of granulosa cell proliferation. Ovulation generally occurs a few hours after the LH surge in rodents, and 24-48 hours after the LH surge in women. The length of the ovarian cycle is approximately 4-5 days in rodents and 28 days in women. The occurrence of the LH surge not only requires high circulating estradiol as an indication of follicle maturation but also a daily signal. Indeed the LH surge arises at a very specific time of day, usually at the end of the resting period, thus in the late afternoon in nocturnal rodents [60][61][62] and the end of the night/early morning in the diurnal rodent *Arvicanthis* [63] and in humans [64]. Such timing requires that the GnRH neurons receive a correct daily signal and the mechanisms by which this is achieved are discussed below.

Most species show additional seasonal cycles in reproductive activity. Indeed, except for the inbred laboratory mice and rats and species like humans living all year long in rather stable conditions, animals living in the wild adapt their breeding activity to the seasonal change in their environment. Early studies have demonstrated the pivotal role of the pineal hormone melatonin in this process [65][66]. Melatonin is a reliable endocrine representation of the seasons since its production at night is proportional to night length. Changes in circulating melatonin by pineal lesion or timed melatonin infusion have profound effect on the occurrence and timing of the reproductive activity. It is now well established that melatonin acts mainly at the pars tuberalis of the pituitary to regulate thyroid stimulating hormone (TSH) synthesis. This TSH in turn acts on the tanycytes located at the basal part of the third ventricle to regulate deiodinases 2 and 3 expression, leading to increased hypothalamic levels of T3 in long day conditions [67]. In male and female seasonal rodents, the higher TSH/T3 signal in spring/autumn increases RFRP and Kp expression and further stimulates the gonadal activity [32][40][68][69][70].

4- Evidence for a circadian control of the pre-ovulatory surge

Most biological functions are synchronized to the daily variation of environmental factors using the recurring light/dark cycle. The mechanisms by which light and dark synchronize biological functions involve the retino-hypothalamic tract (RHT) which transmits the light to the hypothalamic suprachiasmatic nuclei (SCN), locus of the master biological clock in the non-visual light system. SCN neuronal/hormonal outputs then convey the daily cues to downstream central and peripheral structures. The circadian activity of the SCN neurons relies

on a complex molecular system cycling endogenously with a period of about one day [71][72]. This molecular clockwork is composed of transcription-translation loops involving the binding of CLOCK/BMAL1 dimers on E-box to promote transcription of other clock genes whose proteins form PER/CRY dimers which in turn repress their own transcription by competing with the CLOCK/BMAL1 binding. This results in sustained cycles with a period of about 24h (circadian) which are observed for weeks or months in isolated SCN explants or dissociated cells. The SCN circadian activity is synchronized to a period of exactly 24h (diurnal) by the daily change in light intensity perceived by melanopsin-containing intrinsically photosensitive retinal ganglion cells projecting directly to the SCN via the RHT [73][74]. Upon light activation, these ganglion cell terminals release glutamate and pituitary activating cAMP peptide (PACAP) which change the phase of (synchronize) the circadian clock machinery. The CLOCK/BMAL1 dimers not only activate canonical clock gene expression, but also other clock-controlled genes whose promoters display E-boxes and therefore undergo rhythmic expression. This mechanism was first demonstrated for the gene encoding vasopressin, an important output of the SCN clock [75]. Levels of SCN vasopressin mRNA are markedly higher during the day than at night, while in Clock mutated mice the SCN vasopressin rhythm is strongly dampened [76].

Although the master SCN circadian clock is essential in driving biological rhythms, it is now established that secondary clocks located in other central structures and organs are part of a complex a multi-oscillator system [77]. The use of Per2::LUCIFERASE transgenic mice, where the Per2 promoter drives the expression of the luciferase gene, was decisive for the demonstration that non-SCN structures can sustain endogenous circadian oscillations [78]. Although the strength of these secondary clocks is often less than that of the SCN, an

increasing number of studies report that they may play a critical role in the biology of the host structure [79]. In this context, the hypothalamo-pituitary gonadal axis also appears as a functional multi-oscillatory axis since all reproductive structures from the hypothalamic Kp and GnRH neurons down to the ovaries and the uterus display endogenous circadian oscillations of clock genes [80]. Notably, we recently reported that virtually all AVPV Kp neurons express the PER1 protein with a daily rhythm and that isolated Kp-expressing AVPV explants from PER2::LUCIFERASE mice display endogenous estradiol-sensitive circadian oscillations with a period of about 23h [81]. Further, a recent study also reported that RFRP neurons express PER1 with daily variation [82]. Yet, the functional role of these reproductive clocks with regards to the timing of reproduction (ovulation, implantation, parturition) has still to be determined.

The timing of the pre-ovulatory LH surge strongly depends on a functional circadian clock. Indeed, the LH surge not only requires a critical threshold of estradiol produced by the developing ovarian follicles, but it is also gated at the end of the daily resting period. Thus, in female nocturnal rodents like mouse, rat or hamster, the surge of LH occurs at the day(rest)/night(wake) transition of the proestrus stage whereas in the few diurnal mammals investigated the LH surge occurs at the end of the nocturnal resting period. The daily signal driving the LH surge onset arises primarily from the master SCN clock since early experiments of SCN lesions [83] or cuts of the SCN-preoptic area neuronal connection [84] resulted in an impaired LH surge and estrous cyclicity in female rats. Furthermore, female mice carrying mutations of Clock or Bmal1 display disrupted estrous cycles [85][86][87][88][89]. In humans it was reported that women with single-nucleotide polymorphisms in the ARNTL (Bmal1) have more miscarriages and less pregnancies [90].

5. Neuroanatomical and pharmacological evidences of functional connections between the SCN and GnRH neurons

SCN neurons contain various neuropeptides, notably vasopressin and vasoactive intestinal peptide (VIP), whose synthesis displays daily variation. These neurons project to various brain areas to help synchronizing biological function with the time of the day. Early studies have demonstrated that central injection of vasopressin or VIP antagonists (or antibody) reduces the amplitude of LH surge in female rodents, clearly indicating that these peptides may be involved in the daily gating of the LH surge [91][92][93][94][95].

Recent studies indicate that the SCN signal the time of day to GnRH neurons indirectly via vasopressinergic fibers projecting to the AVPV Kp neurons [96][97]. Anterograde tracing studies show that vasopressin-containing axons originating from the SCN make appositions to Kp neurons and these neurons express V1a receptors. Notably, vasopressin is released with a peak coinciding with the onset of the LH surge [98]. The vasopressin input to Kp neurons is sensitive to estradiol since estradiol treatment significantly increases the number of vasopressin terminal appositions on individual Kp neurons [97] and the rhythm in V1a mRNA is abolished in ovariectomized animals [81]. Furthermore, vasopressin activation of Kp neurons is critically dependent on circulating estradiol as vasopressin no longer activates Kp neurons in ovariectomized mice, an effect that is fully restored by estradiol treatment [99]. A recent study reported that intracerebroventricular administration of vasopressin in female

Syrian hamsters activates Kp neurons similarly in the early or late part of the day, while in the same animals GnRH neurons are activated only late in the day [96]. Altogether, these results are consistent with the hypothesis that Kp neurons located in the rodent AVPV receive daily information from the SCN via a vasopressinergic monosynaptic pathway, a signal which is modulated (gated) by circulating estradiol. Vasopressinergic fiber projections are also found on RFRP neurons, but central administration of vasopressin does not appear to regulate RFRP neurons [82].

A significant role of the SCN-derived VIP output in female reproduction should not be excluded. Indeed, VIP neurons project onto GnRH neurons in a sexually dimorphic manner, with female rats exhibiting higher VIPergic innervation than males [100]. On the contrary, very few or no VIP terminals were found to make appositions on to Kp neurons [96][97]. Notably, recent studies reported that SCN-derived VIP neurons project to RFRP neurons and that central administration of VIP decreases RFRP neuronal activity in the afternoon, but not in the morning, of the proestrous stage [82]. Therefore, it seems likely that a SCN-derived VIPergic signal regulates RFRP activity. However, RFRP cells do not appear to express VIP receptors, suggesting that VIP regulation of RFRP neurons may be indirect [82].

Thus, rodent studies indicate that the SCN clock predominantly uses a vasopressin, estradiol dependant, signal towards AVPV Kp neurons and a VIP signal towards RFRP neurons, via a yet undetermined target, to forward daily cues to the reproductive axis (figure 1). Further, it cannot be excluded that putative secondary clocks operating within the Kp and RFRP neurons may play a role in timing their cellular activity.

6. Role of Kp and RFRP in relaying circadian signal to GnRH neurons

The putative role of Kp and RFRP neurons as a relay between the SCN clock and the daily GnRH/LH surge presumes that their neuronal activity displays daily changes coordinated with the onset of the LH surge.

Actually, under high circulating estradiol condition (either in proestrus or in ovariectomized/estradiol implant conditions) Kp neuronal activity (as seen by c-FOS activation) and Kiss1 expression are significantly increased about 3 hours before lights off, thus 2 hours before the LH surge in female rodents [81][96][101][102]. On the opposite, Kp immunoreactivity is markedly but transiently decreased suggesting a release of the Kp peptide at the same time [102]. Under low circulating estradiol conditions, in diestrus or in ovariectomized animals, the daily variation in neuronal activity, Kiss1 mRNA and Kp immunoreactivity is abolished or strongly dampened [81][101][102]. These data indicate that in female rodents, Kp neurons are activated at the day/night transition (end of the resting period) only during the proestrus stage, in coordination with the LH surge (figure 1). Intracerebroventricular injection of vasopressin induces c-FOS expression in Kp neurons and increases Kiss1 mRNA [96] while vasopressin incubation of hypothalamic sections activates Kp neuron firing rate [99]. Altogether these data demonstrate that only under high circulating estradiol (when oocytes are mature enough to be released), Kp neurons can be activated by the SCN-derived vasopressin in order to increase Kp synthesis and release. Given the highly

potent stimulatory effect of Kp on GnRH release and LH/FSH secretion, this SCN-driven activity of Kp neurons is pivotal for the daily gating of the LH surge. All these studies have been performed in nocturnal female rodents, thus similar investigations should be performed in diurnal mammals to assess whether Kp neuronal activity is increased at late night together with the LH surge.

RFRP neurons also display a daily rhythm in neuronal activity in the various female rodents investigated [32][103][104]. Unlike Kp neurons, however, the number of c-FOS expressing neurons is decreased at end of the day of the proestrus stage, thus at the time of the LH-surge (figure 1). Similarly, in ewes RFRP expression is reduced during the pre-ovulatory period [105]. In the female hamsters, this daily decrease in RFRP neuronal activity is observed not only at the proestrus, but also at the diestrous stage thus indicating that in contrast to Kp neurons, the daily regulation of RFRP neurons does not depend on circulating estradiol [32]. Given the robust inhibitory effect of RFRP-3 on the GnRH/LH pre-ovulatory surge, these findings point towards a specific daily rhythm in RFRP expression and release in females, which serves to down-regulate the inhibitory RFRP activity at the time of the LH-surge.

Altogether, it is tempting to speculate that the SCN master clock uses two different nervous pathways (vasopressin and VIP) to drive a coordinated increase of the stimulatory Kp neurons and decrease of the inhibitory RFRP neurons to allow a full stimulation of the GnRH/LH surge at the end of the resting period (figure 1).

7- What happens when the daily activity goes wrong?

Concept of shift work

The modern 24h-functioning society requires an increasing number of employees to work outside of the natural active period, in shifted conditions. According to the International Labor Organization (ILO; 1990), working in shifts is “a method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work of individual workers”. Shift work and night work cover a multitude of realities: different time systems called 2x9, 3x8, 4x8, 5x8, 2x12 hours with variability resulting from different choices made by the employer's company.

Nowadays in industrial countries, 20-30 % men and 15-20% women experience shift work or work at night [106], and this is an expanding phenomenon with a particularly significant increase among women under 30 years. One difficulty to define shift/night work comes from variable definitions, even within the European Union. Thus, in France, night work is defined as any work between 9 pm and 6 am; in Germany it is 2 hours of the daily work between 11 pm and 6 am; in Italy it is a minimum of 7 consecutive hours between 0 am and 5 am; in Belgium it is performed between 8 pm and 6 am; and in the United Kingdom, it is 3 hours of the daily work between 11 pm and 6 am. Moreover, shift work can be defined by a number of periods, duration of the periods, shift structure (continuous or not), start and end time of work, and overlapping time between shifts.

Impact on health in general

An increasing number of studies reports that shift work or night work is often associated with increased risks of developing cardiovascular/metabolic/gastro-intestinal disorders, some types of cancer, and mental disorders including depression and anxiety [107][108][109]. Hence, in 2007 shiftwork was reclassified from a possible to a probable human carcinogen (class 2A) by the International Agency for Research on Cancer.

Many epidemiological studies and meta-analyses have reported a correlation between shift/night work and risks of metabolic disorders and cardiovascular diseases such as obesity or overweight, diabetes, hypertension, dyslipidemia, or metabolic syndrome. Bøggild and Knutsson (1999), who analyzed 17 studies (between 1949 and 1998), evaluated the excess risk at 40% for ischemic heart disease in shift/night workers compared to day workers (relative risk was ranging from 0.4 to 3.6, with a majority between 1 to 2) [110]. Ten years later, Frost et al. published a new review (from 16 epidemiological studies done between 1972 and 2008) which reported a limited epidemiological evidence for a correlation between shift/night work and ischemic heart disease [111]. More recently, a large meta-analysis (34 studies published between 1983 and 2011, including more than two million people) indicated that shift/night work is associated with a significant increase in myocardial infarction and coronary events with or without adjustment for other risk factors [112]. Since then, four other epidemiological studies have indicated an increased risk of coronary events and

cardiovascular disease mortality after 5 years of shift/night work [113][114][115][116]. A causal link between shift/night work and weight gain/high body mass index is often reported, notably after 5 years, suggesting that shift/night work is a risk for type 2 diabetes [117]. Indeed, a retrospective study on 6413 male shift/night workers showed an increased risk of impaired glucose tolerance (even for workers with normal and stable bodyweight) compared to day workers [118]. Similarly, a recent meta-analysis reported an increased risk of 1.09 between shift/night work and type 2 diabetes [119]. Shift/night work is also often associated with chronic stress and a significant impact on cortisol (in humans) or corticosterone (in rodents) is well documented [120][121][122][123][124]. This is important because these hormones play a major role in the circadian resynchronization of the central and peripheral clocks in a chronic jet-lag context [122]. Given the importance of the circadian system in the regulation of female reproduction, and given the fetal exposure to the maternal daily rhythms in temperature, hormones and metabolic cues, female shift workers may display reproductive dysregulations. Indeed a few studies have reported increased risk of irregular menstrual cycles, endometriosis, miscarriage, low birth weight or pre-term delivery in women in shift/night work conditions [125][126][127]. Notably, an animal study showed that the functioning of fetal clocks depends on maternal hormones thus suggesting that maternal circadian disruption during pregnancy may lead to fetal SCN and peripheral clock desynchronisation [128].

Modeling shift work in rodents

Shift work is a very complex situation and therefore it is difficult to design animal model conditions that truly mimic human shift work which is often associated with potential confounding factors (diet, social stress, sleep disturbance, use of psychostimulants). Furthermore, most studies are carried out on nocturnal animals (rats, mice, hamster) while humans are diurnal. Apart from melatonin, whose secretion is always at night, other hormones (cortisol/corticosterone, glucose, leptin, gonadotropins) and many biological functions (food intake, sleep/wake, cardiac functions, vigilance) have opposite rhythms between diurnal and nocturnal species. Moreover, for these studies male rodents are mainly used to avoid an effect of the female reproductive cycles in the measurement of the circadian disturbances. Yet, animal studies are essential for understanding the cellular and molecular mechanisms underlying circadian perturbations. A recent review listed four relevant models that use altered timing of either food intake, activity, sleep or light exposure, or a combination of several [129].

Few animal studies have investigated alteration in fertility or LH surge timing after a shift in the light/dark cycle or photoperiod. One study in female Syrian hamsters reported that after a 3h phase advance, the LH surge is not fully resynchronized to the new dark onset even after 3 days, but when they are submitted to a 3h phase delay, the LH surge is synchronized more rapidly [130]. Further, photoperiod lengthening was associated with similar shifts in locomotor activity and the LH surge in female hamsters [62]. In mice, exposure to either phase advances or delays at the beginning and throughout pregnancy leads to a significant decrease in pregnancy success [131]. Finally, an *in vitro* study reported that the ovarian clock was not fully resynchronized 6 days after a 6h phase advance in PER2::LUCIFERASE mice [132].

Taking advantage of a new blood LH micro-assay [133], we recently investigated the effect of a single 10h phase advance on circulating LH and the estrous cycle length in C57BL/6 female mice (Bahougne et al. unpublished). After the 10h phase advance, the first two estrus cycles were longer and irregular and were restored at the third estrous cycle (with an average of 4.9 ± 1.2 days for 9 mice). Figure 2 illustrates the shift of the pre-ovulatory LH surge in one representative mouse submitted to a 10h phase advance: after the shift, the LH surge was delayed by 2-3 h at the first proestrus, displayed 2 peaks at the second proestrus, and was finally synchronized to the light/dark transition at the third proestrus. Altogether these preliminary data clearly indicate that a single phase shift can alter the timing of the LH surge and the length of the estrous cycles.

Conclusions and Summary

In female mammals the timing of ovulation depends on a large and transitory LH surge gated by high circulating estradiol produced by mature oocytes and a daily signal provided by the biological clock located in the suprachiasmatic nuclei. As shown in female rodents, in which the LH surge occurs at the end of the daytime resting period in proestrus, the suprachiasmatic nuclei appear to use two different pathways to forward daily signals to the reproductive system. Vasopressin neurons project to kisspeptin neurons to increase their activity precisely at the end of the day of proestrus, while vasoactive intestinal peptide decreases the activity of RFRP neurons at exactly the same time of the day. Given that kisspeptin stimulates and RFRP

inhibits GnRH neurons and LH secretion, this coordinated activation of kisspeptin neurons and inactivation of RFRP neurons by the circadian clock allows a precise gating of the pre-ovulatory LH surge at the end of the resting period. Epidemiological investigations together with animals studies now indicate that circadian disruption observed when the light/dark cycle is acutely (jet-lag) or chronically (shift work) shifted impairs the timing of the reproductive cycles.

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Figure 1

Model of a neuronal network controlling the gating of the LH surge at the day/night transition of the estrous stage in female mice. Neuronal activity of the circadian biological clock located in the suprachiasmatic nuclei (SCN) is synchronized by the daily change in light and dark. SCN vasopressin (VP) neurons project to kisspeptin (Kp) neurons located at the anteroventral periventricular nuclei (AVPV) and VP is released at the end of the light phase to activate Kp neurons (c-Fos expression) only at the proestrus stage (due to the permissive effect of high circulating estradiol at this stage). SCN vasoactive intestinal peptide (VIP) project to (Arg)(Phe)related peptide (RFRP) neurons located at the dorsomedial hypothalamus (DMH) and VIP inhibits, possibly via an indirect pathway, RFRP neuronal activity (cFOS expression) at the end of afternoon of both proestrus and diestrus stages. Kp exerts a strong stimulation on the GnRH-induced LH release whereas RFRP-3 reduces the elevated LH secretion. Thus, the SCN appears to induce a coordinated increase of the stimulatory Kp (via VP) and decrease of the inhibitory RFRP-3 (via VIP) in order to allow a full activation of LH secretion at the light/dark transition on the day of proestrus. Adapted from Henningsen et al., 2017.

Figure 2

Timing of the LH surge in one mouse submitted to a 10h phase advance. The adult female mouse was adapted to a 12h light/12dark cycle with light on at ZT (zeitgeber) 0 and light off at ZT12. The estrous cycle was followed by vaginal smears and LH levels were measured by radioimmunoassay in 4 μ l tail blood sampled every hour. Before the phase advance, the LH surge (grey dotted line) occurs at the very end of the light phase (ZT11-ZT12). After the 10 h phase advance, estrous cycles and LH secretion were followed. The LH surge of the first proestrus stage after the shift occurs 2 hours after lights off (ZT14); the surge displays two peaks (at ZT14 and ZT12) at the second proestrus stage; and at the third proestrus after the shift the LH surge is finally gated at the new day/night transition (ZT10-ZT11). Preliminary data from Bahougne and Simonneaux (unpublished)