REPRODUCTION
REVIEW

Circadian rhythms and reproduction

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Abstract

There is a growing recognition that the circadian timing system, in particular recently discovered clock genes, plays a major role in a wide range of physiological systems. Microarray studies, for example, have shown that the expression of hundreds of genes changes many fold in the suprachiasmatic nucleus, liver heart and kidney. In this review, we discuss the role of circadian rhythmicity in the control of reproductive function in animals and humans. Circadian rhythms and clock genes appear to be involved in optimal reproductive performance, but there are sufficient redundancies in their function that many of the knockout mice produced do not show overt reproductive failure. Furthermore, important strain differences have emerged from the studies especially between the various *Clock* (*C*ircadian *L*ocomotor *O*utput *C*ycle *K*aput) mutant strains. Nevertheless, there is emerging evidence that the primary clock genes, *Clock* and *Bmal1* (*B*rain and *M*uscle *A*RNT-*l*ike protein 1, also known as *Mop3*), strongly influence reproductive competency. The extent to which the circadian timing system affects human reproductive performance is not known, in part, because many of the appropriate studies have not been done. With the role of *Clock* and *Bmal1* in fertility becoming clearer, it may be time to pursue the effect of polymorphisms in these genes in relation to the various types of infertility in humans.

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Introduction

Animals have developed a range of strategies to ensure optimal chances for survival, among them, processes for ensuring the optimal timing of reproductive function. Ultimate reproductive success is built upon the production of offspring during mild weather and with a growing period during optimal food availability. Many animals have evolved a mechanism for seasonal fertility to achieve this result to ensure that parturition occurs from spring to early summer. In animals where the gestation length is short (e.g. hamsters and other small mammals; Reiter 1980), sexual development and reproductive competence are triggered by changes in their environment that signal the onset of spring. In larger animals, with longer gestation lengths (e.g. ruminants; Lincoln 2002), the onset of reproductive function occurs in late summer and autumn resulting in gestation over the winter months and parturition in spring. There are many possible environmental cues that animals can use to induce and arrest seasonal fertility, including temperature, rainfall and food availability (including specific plants). However, these environmental variables can be notoriously unreliable and would pose a risk for animals that relied entirely on them for timing of reproductive functions. Changes in day length as the seasons progress provide a far more robust marker of the 'time of year' and it has been found that this is the predominant cue for the timing of seasonal breeding. The environmental signal for changes in reproductive competency is the change in the time of sunrise and sunset, which together with an endogenous timing system, controls the daily secretion of the pineal hormone melatonin (Reiter 1980). The daily rhythm of melatonin production, in conjunction with other circadian neural and hormonal signals, regulates the onset of seasonal fertility and subsequent parturition. The changing patterns of melatonin secretion are also responsible for controlling the onset of puberty, ensuring, e.g. that late born animals do not become pregnant and deliver offspring at an inappropriate time of the year (Foster *et al.* 1988).

In this review, we will discuss the role of the endogenous timing system and environmental light in reproductive function in a range of species. Until recently, this would have meant a strong emphasis on the central rhythm generator, the suprachiasmatic nucleus (SCN) and its control of pineal melatonin secretion. The recent discovery of clock gene transcription factors in a wide range of tissues outside

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the brain has suggested that cellular rhythmicity may have even broader biological implications, including reproductive biology.

The circadian timing system

The vertebrate timing system has three main components: a means of sampling the environment, a self-sustaining clock and an output pathway for communicating the information to peripheral organs and tissues. The key environmental signal, light, is detected by the retina and its presence or absence is signalled via one direct and at least two indirect neural pathways to the SCN (Morin *et al.* 2005). Excitatory amino acids mediate the light information from the retina, while other inputs from the intergeniculate leaflet and raphe nuclei utilise GABA (gamma amino butyric acid), neuropeptide Y and serotonin. Understanding the role of the transmitters in the neural pathways innervating the SCN is particularly important, since they may provide a means to manipulate the SCN pharmacologically, but it is beyond the reproductive focus of this review (see instead Morin & Allen 2006).

The importance of the SCN for circadian rhythm generation is apparent from the impact of lesion experiments. Discrete SCN lesions result in the loss of hormonal and behavioural circadian rhythms and the inability to respond to changes in the external environment (Moore & Eichler 1972, Stephan & Zucker 1972). The SCN, in addition to its role as a relay station for the light signals from the retina, is a self-sustained oscillator and so in the absence of a rhythmic environment (e.g. continuous darkness), animals, including humans, continue to express hormonal and behavioural rhythms, but with a period usually differing slightly from 24 h. When isolated and cultured *in vitro*, the SCN maintains a robust near 24 h rhythm of neuronal firing (Green & Gillette 1982), further reflecting the endogenous nature of the rhythmicity.

The SCN transmits information to the rest of the body through both humoral and neural pathways. At least three proteins (arginine vasopressin, TGF alpha and prokineticin 2) are secreted directly into the cerebrospinal fluid by the SCN, and control temperature and locomotor activity (Schwartz & Reppert 1985, Kramer *et al.* 2001, Cheng *et al.* 2002). The neural connections between the SCN and the peripheral targets are extensive and include the multi-synaptic pathway, which controls the pineal gland (Teclemariam Mesbah *et al.* 1999), adrenals (Buijs *et al.* 1999), thyroid (Kalsbeek *et al.* 2000), heart (Scheer *et al.* 2001), liver (la Fleur *et al.* 2000) and pancreas (Buijs *et al.* 2001). Within the brain, SCN projections to the magnocellular pre-optic nucleus (POMA) provide control over the timing of the release of gonadotrophin-releasing hormone (GnRH) (van der Beek *et al.* 1997*a*, Palm *et al.* 1999, 2001). The SCN signals the time of day to the whole organism through this repertoire of signalling pathways to the rest of the brain, endocrine glands and other peripheral tissues, facilitating the entrainment of the physiological systems to the external photoperiod and maintaining synchrony between organs.

Molecular timing system

The cellular rhythmicity of the SCN is generated by a series of interlocking positive and negative feedback gene transcription and translation loops (Fig. 1). The primary positive arm of the rhythmicity (Reppert & Weaver 2002) is generated by two transcription factors, *Clock* (circadian locomotor output cycle kaput) and *Bmal1* (brain and muscle ARNT-like protein 1, also known as *Mop3*). After transcription and translation, the CLOCK and BMAL1 proteins form a heterodimer and return to the nucleus to bind to a specific enhancer region (E-Box, CACGTG; Hogenesch *et al.* 1998) in the promoters of the period genes *per1* and *per2* and cryptochrome genes *cry1* and *cry2* and drives their transcription (Gekakis *et al.* 1998, Jin *et al.* 1999, Kume *et al.* 1999). The translated CRY and PER proteins complex with casein kinase 1ε and translocate into the nucleus to interfere with the CLOCK/BMAL1 heterodimer complex on the E-boxes in their own promoter regions, thus blocking their own transcription. The degree of phosphorylation of the PER and CRY proteins by casein kinase 1ε (Lowrey *et al.* 2000) alters the protein stability and hence the speed of the feedback cycle. The CLOCK/BMAL1 heterodimer also induces the transcription of *Rev-erbα* and *Rora* genes, which interact with *Rev-erb*/*Ror* elements (RREs) in the promoter of *Bmal1*, repressing and driving its transcription respectively (Preitner *et al.* 2002, Sato *et al.* 2004).

A further negative loop is generated by the transcription of the *dec1* (*sharp2*/*stra13*) and *dec2* (*sharp1*) genes, which are also driven by CLOCK/BMAL1 via E-boxes in their promoters (Grechez-Cassiau *et al.* 2004). DEC1 and DEC2 proteins may block circadian gene expression, in part by the formation of a non-functional heterodimer with BMAL1 (and hence inhibiting the expression of all genes dependent on an E-box), as well as playing a role in light induction of genes in the SCN (Honma *et al.* 2002). Gonadotrophins and insulin are known to induce *dec1* gene expression and this may provide an avenue for the reproductive system and pancreas to influence circadian timing both centrally and in other peripheral tissues (Yamada *et al.* 2003, 2004).

While these interlocking feedback loops are critical for the generation of rhythmicity in the SCN, it is also now clear that cells in a wide range of tissues (e.g. liver, adrenal, kidney, heart, pancreas, muscle) also express clock genes (Hastings *et al.* 2003), although the phasing of the rhythms in various tissues may be different from the SCN rhythm (Yamazaki *et al.* 2000). Of particular interest, in the context of this review, is that the testis is one of the few tissues to show an absence of cyclic clock

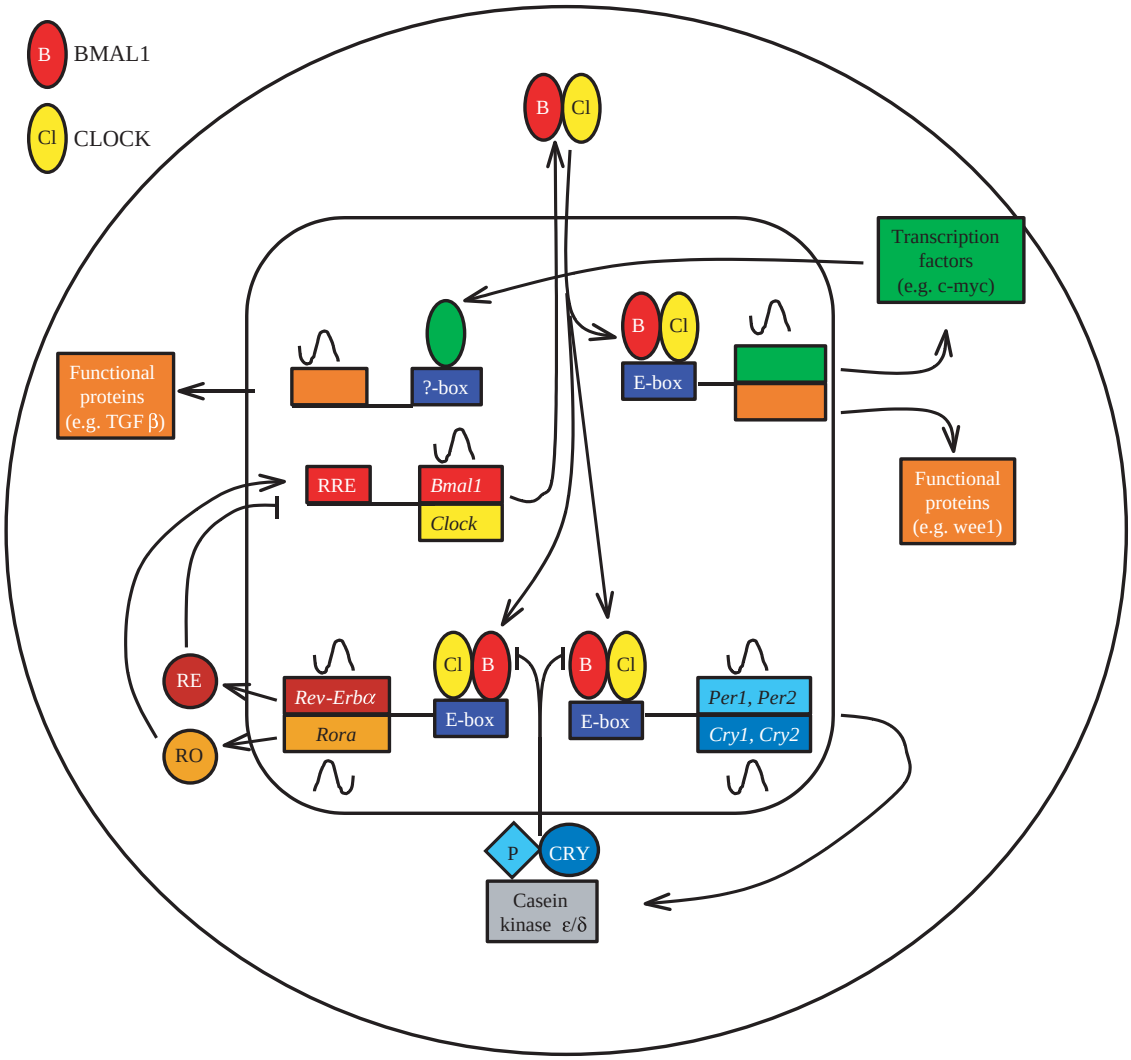

Figure 1 The molecular clock in mammalian suprachiasmatic nucleus and other tissues. The transcription factors, CLOCK (Cl) and BMAL1 (B), heterodimerise, re-enter the nucleus and initiate transcription of the period genes (*per1* and *per2*) and the cryptochrome genes (*cry1* and *cry2*) through an E-box (CACGTG). The period and cryptochrome proteins complex with casein kinase and re-enter the nucleus. The degree of phosphorylation of PER by casein kinase alters its stability and thus regulates the amount of protein available to re-enter the nucleus. The PER/CRY complex inhibits the action of the CLOCK/BMAL1 on the E-box and so inhibits its own transcription. CLOCK/BMAL1 also drives the expression of *rev erb* α and *Rora* in opposite phases and their proteins compete with the RRE element on the promoter of the *Bmal1* gene. As a result of these interactions, a transcription/translation loop is established that takes approximately 24 h to complete. The rhythmic production of the CLOCK/BMAL1 heterodimer has an additional function of driving other genes that have the appropriate CACGTG sequence(s) in their promoters, such as the transcription factors *c-myc, dbp, tef,* etc. as well as functional proteins like *wee1, pai-1, avp,* etc. The rhythmic expression of the transcription factors can in turn result in the rhythmic expression of their target genes, e.g. *tgfβ*.

gene expression (Morse *et al.* 2003). In the testes, clock genes may still play a role in spermatogenesis, as there is a change in the level of PER1 protein generated over the course of sperm maturation (Alvarez *et al.* 2003, Bittman *et al.* 2003). There is also some evidence for the rhythmicity of clock gene expression in the hamster testis (Tong *et al.* 2004), but the control of this expression is atypical. Hamsters undergo marked testicular recrudescence in long day length and during this period of testicular growth, only the *per1* gene was cyclically expressed, particularly within the seminiferous tubules, while during testicular regression, only *Bmal1* expression was cyclic, again primarily within the seminiferous tubules (Tong *et al.* 2004).

In addition to the cyclic expression of clock gene transcription factors, a significant proportion of the genome is also expressed rhythmically in both the SCN and peripheral tissues. Microarray studies of the liver and heart have revealed that more than 300 genes are rhythmically expressed (Akhtar *et al.* 2002, Panda *et al.* 2002, Storch *et al.* 2002, Oishi *et al.* 2003). Some of these genes possess the appropriate circadian E-box sequences in the promoter regions that respond directly to the CLOCK/BMAL1 heterodimer (e.g. *wee1*), while

others are rhythmically induced by other tissue-specific clock-controlled transcription factors (e.g. *rev erbα*, *dbp*, *c-myc*). Since rhythmic expression of many of these 'clock-controlled genes' is tissue-specific, it has been suggested that other upstream and downstream regulator sequences provide specificity and fine tuning of circadian gene expression (Munoz *et al.* 2002).

Circadian rhythm phenotype following clock gene disruption

Disruption of clock gene expression by mutation or gene knockout should result in profound changes in circadian rhythmicity. The following section discusses the clock gene knockout and mutant mice that have been generated and the impact of the gene disruption on behavioural rhythmicity (predominantly wheel running). An appreciation of the varying impact of these manipulations is helpful in understanding their potential role in other physiological systems, including reproductive function.

Period (per) *genes*

Three different *per1* knockout mouse lines have been produced to date (Albrecht *et al.* 2001, Cermakian *et al.* 2001, Bae & Weaver 2003) and all three maintain apparently normal entrainment of running wheel activity to a light:darkness cycle (LD). However, underlying circadian timing system deficits emerge in wheel running rhythmicity in constant darkness and two of the *per1* mutant lines respond to morning and evening light pulses in constant darkness with appropriate phase advances and delays respectively. One line, however, failed to respond to morning light pulses with a phase advance in running activity (Albrecht *et al.* 2001).

Two *per2* knockout mouse lines have been developed (Zheng *et al.* 1999, Bae & Weaver 2003) and like the *per1* knockout mice, there was apparent normal entrainment to LD cycles in both strains. One *per2* knockout mouse responded to a morning light pulse, but not to an evening light pulse (Albrecht *et al.* 2001), while the other line responded appropriately to both morning and evening light pulses (Bae & Weaver 2003).

The *per3* knockout mice showed only small changes in behavioural rhythmicity, with a 0.5-h shortening of the period in constant darkness (Shearman *et al.* 2000). This and other evidences suggest that *per3* is not a major component of the circadian timing system.

The *per1*/*per2* double knockout mice show a complete and immediate loss of circadian rhythms when placed in constant darkness (Bae *et al.* 2001, Zheng *et al.* 2001), confirming the likelihood of *per1* and *per2* compensating for the loss of function in single knockouts. The loss of these genes results in the loss of PER–CRY complex formation, as PER3 forms only weak interactions (and hence poor ability to translocate) with other clock proteins in the absence of PER1 (Lee *et al.* 2004). Mice with both *per1* and *per3* or *per2* and *per3* disrupted were similar to single *per1* and *per2* knockout animals (Bae *et al.* 2001), further suggesting that *per3* is functionally less important than the other period genes.

Cryptochrome (cry) *genes*

The *cry1* knockout mice have a free-running period of wheel running activity approximately 1 h shorter than wild-type mice in constant darkness and a reduced *per1* response to light in the SCN during the dark phase (Vitaterna *et al.* 1999). However, the ability of light to induce expression of *per2* was not effected by the loss of *cry1* and the animals still show a daily oscillation of *per1* and *per2* within the SCN. The *cry2* knockout mice, on the other hand, have a longer free-running period of approximately 25 h in constant darkness and while there is a reduced induction of *per1* in response to a light pulse, exposure to a saturating light pulse resulted in a larger phase shift in behaviour rhythms than in the wild-type mice (Thresher *et al.* 1998). The *cry2* knockout mice also maintain a rhythm in *per1* and *cry1* expression within the SCN under normal conditions. The loss of both *cry1* and *cry2* results in a complete loss of behavioural rhythmicity in constant darkness (van der Horst *et al.* 1999), suggesting that they can substitute for each other to rescue circadian function. The *cry1*/*cry2* double knockout also shows a loss of rhythmic *per1* mRNA expression in the SCN, as well as a failure of light-induced expression of *per1* (while *per2* expression remains intact). There was a loss of rhythmic expression of clock genes (*dec2*, *per1*) in the liver of *cry1*/*cry2* knockout mice and either higher or lower expression of genes involved in a wide variety of functions, including metabolism, cell signalling, immune and transcription factors (Oishi *et al.* 2003).

Period and cryptochrome genes

The consequences of combining multiple *per* and *cry* knockout lines have been investigated with results similar to those seen in single knockout animals (Oster *et al.* 2002, 2003). While a *per2* knockout mouse eventually looses its circadian rhythm when placed into constant darkness, when combined with the loss of *cry2* function (*per2*/*cry2* knockout mouse), apparent normal rhythmicity is detected. The *per2*/*cry1* knockout mouse, on the other hand, shows complete loss of rhythmicity in constant darkness. The *per1*/*cry1* knockout mouse can be entrained to a LD cycle and in constant darkness-free runs with a period of 23.7 h (similar to wild-type), while *per1*/*cry2* knockout mice entrain to LD conditions, but become arrhythmic in constant darkness. Interestingly, as the *per1*/*cry2* knockout mice age, they loose their ability to maintain entrainment to a LD cycle. These studies together suggest that at least one complex, either

of PER1 and CRY1 or PER2 and CRY2, is needed for a functional circadian timing system.

While the behavioural effects of *per* and *cry* gene disruption have been evaluated, the downstream peripheral effects have not been as thoroughly investigated. The gene expression in peripheral tissue of *per* and *cry* null mice has been investigated (primarily in the liver, kidney and muscle), showing results that correlate with the behaviour rhythms; animals with a mildly disrupted behaviour rhythm (*per1*, *per2*, *per1*/*cry1*) show some disruption in peripheral tissue gene expression patterns (Zheng *et al.* 2001, Oster *et al.* 2003), while animals with a more severe disruption in behaviour patterns (*per1*/*cry2*, *cry1*/*cry2*, *per1*/*per2*) show a far greater level of rhythmic gene expression disruption (Zheng *et al.* 2001, Oishi *et al.* 2003, Oster *et al.* 2003).

Clock gene

Unlike the *per* and *cry* knockout mice, the *Clock*$^{\Delta 19}$ mutant mouse was generated by ENU mutagenesis with an A→T mutation causing the skipping of exon 19 and the loss of 51 amino acids in the C-terminal region of the translated protein (King *et al.* 1997*a*, 1997*b*). As a result, while the CLOCK$^{\Delta 19}$ protein can form a dimer with BMAL1 and bind to E-boxes, it is unable to drive transcription (Gekakis *et al.* 1998). The *Clock*$^{\Delta 19}$ mutant mouse is entrained to the LD cycle, although there is often elevated wheel running activity in the light period (Vitaterna *et al.* 1994). Upon transfer to constant darkness, rhythmicity often persists for a period of 26–27 h, although many animals eventually become arrhythmic. *Clock* gene expression patterns in the liver, heart, kidney and muscle of the *Clock*$^{\Delta 19}$ mutant mouse are either arrhythmic or severely dampened (Oishi *et al.* 2000, Noshiro *et al.* 2005). Microarray studies have also shown that a wide range of clock-controlled genes are disrupted in peripheral tissues (Oishi *et al.* 2003).

Bmal1 gene

The *Bmal1* knockout mouse has reduced activity levels in conjunction with an inability to consolidate activity to the dark period (Bunger *et al.* 2000), changes in sleep architecture and an increase in daily sleep time (Laposky *et al.* 2005). Exposure to constant darkness results in immediate loss of any significant rhythmic wheel running behaviour. There is reduced expression and loss of rhythmicity in *per1* and *per2* in the SCN, while the expression of the clock-controlled gene *dbp* is down-regulated and arrhythmic in the liver (Bunger *et al.* 2000).

Casein kinase 1ε gene

The observation of an unusually short free-running period in constant darkness in a hamster led to the generation of the Tau mutant hamster. Subsequent breeding from the individual uncovered an endogenous wheel running activity period of 22 h in heterozygous and 20 h in homozygous animals (Ralph & Menaker 1988). The mutation was subsequently located in the gene for the enzyme casein kinase 1ε (Lowrey *et al.* 2000), which phosphorylates a wide range of proteins including the period proteins, leading to their degradation through the ubiquitination pathway. The failure of casein kinase 1ε to mark PER and CRY proteins for degradation apparently facilitates their early concentration-dependent inhibition of the CLOCK/BMAL1 induction of genes, resulting in a faster circadian cycle.

Clocks and reproduction

The preceding sections of this review discussed the molecular genetics of the circadian timing system and the impact of mutations and gene knockout on behavioural rhythms. In the following section, we discuss the evidence for circadian rhythm influences on reproductive function and the studies reported to date on the reproductive biology of animals with a disrupted circadian timing system.

The luteinizing hormone (LH) surge

There has been considerable research on the circadian gating of the pro-oestrus LH surge in laboratory animals (Barbacka-Surowiak *et al.* 2003). During the follicular phase of the ovarian cycle, oestradiol gradually increases in an environment of increased LH pulsatility. As the dominant follicle continues to grow and develop, there is a change from relative inhibition of GnRH secretion to a positive concerted stimulation, resulting in the surge release of LH from the pituitary and subsequent ovulation of mature oocytes. In mice and rats, the LH surge commences around 1600 h with ovulation occurring at midnight. The role of a physiological timing system in this event was first demonstrated by the administration of phenobarbital in the afternoon of pro-oestrus in both hamsters and rats. The expected LH surge and ovulation were blocked, but only delayed by 24 h, since the following afternoon there was a normal LH surge in animals treated with the drug (Everett & Sawyer 1950). Lesions of the SCN results in the loss of the gating response to elevated oestrogen and hence the cessation of ovulation in rats (Mosko & Moore 1979), but not in sheep (Scott *et al.* 1995), suggesting that different mechanisms may be present in different species. Nevertheless, the SCN is known to project to GnRH-positive neurons in the hypothalamus and the region also expresses oestrogen receptors (van der Beek *et al.* 1993, 1997*b*, de la Iglesia *et al.* 1995, Watson *et al.* 1995), providing a possible pathway for the steroid to influence the timing of the ovulation.

Immortalised GnRH neuronal cells (GT1-7) maintain ultradian rhythms of GnRH secretion *in vitro* and express the full complement of clock genes rhythmically (Chappell *et al.* 2003, Gillespie *et al.* 2003, Olcese *et al.* 2003), especially after serum shock. Transfection of the GT1-7 cells with $Clock^{\Delta 19}$ to overexpress a mutated form of CLOCK with defective transcriptional activity significantly decreased GnRH pulse frequency, while overexpression of *cry1* increased GnRH pulse amplitude without altering the frequency (Chappell *et al.* 2003). These results provide compelling evidence that the SCN as well as endogenous rhythm systems in other parts of the hypothalamus are of critical importance for the timing of the LH surge in rodents.

In humans, evidence for a substantive role of circadian rhythmicity in the timing of LH surge and ovulation is scarce. Part of the reason for this is that intensive, frequent blood sampling, hormone analysis and direct observations of the timing of the ovulatory events have not been conducted. However, based upon infrequent blood sampling, it has been estimated that the LH surge in women occurs between midnight and 0800 h (Cahill *et al.* 1998, Kerdelhue *et al.* 2002), with ovulation occurring either 24 (Vermesh 1987) or 36–48 h later (Wilcox *et al.* 1995, Khattab *et al.* 2005). It is not clear if this wide window is due to a true lack of precision in the timing of ovulation or the inadequate sampling intervals. Since humans are diurnal animals, it is interesting to note that a diurnal rodent, *Arvicanthus niloticus*, also initiates its LH surge in the latter half of the dark phase and normally mates in the morning just prior to dawn (McElhinny *et al.* 1999).

Timing of intercourse

Nocturnal female rodents express overt behaviour that stimulates the males to copulate in close synchrony with ovulation at middark. A delay in insemination for 4–6 h following ovulation can significantly impair the viability of embryos in mice (Sakai & Endo 1988). In humans, there is a strong circadian component to intercourse, with the major peak of sexual activity in the late night (Palmer *et al.* 1982, Refinetti 2005) and a minor peak in the early morning, especially on weekends (Palmer *et al.* 1982). Humans are probably unique in copulating throughout the ovarian cycle and to our knowledge, no studies have been conducted to determine whether the time of day of intercourse in relation to the time of day of ovulation alters fertility outcomes. The human female reproductive 'window of opportunity' appears to be larger than the mouse, with fertility beginning 3–6 days pre-ovulation (Wilcox *et al.* 1995, Khattab *et al.* 2005) and ending at the time of ovulation, as changes in cervical mucus generate a barrier to sperm entry into the uterus preventing fertilisation (Katz *et al.* 1997). Since the actual time of the shedding of ova in humans is not known with any precision, it is difficult to know whether the low human fertility compared to other species is in part due to insemination and fertilisation at inappropriate times of day or other factors.

Ovary

To our knowledge, there are no published studies on clock gene expression or rhythmicity of other genes in the ovary. However, one of the genes implicated in the accessory feedback loop underpinning cellular rhythmicity, *dec1* (*sharp2*), is expressed in the rat ovary and is induced temporarily by eCG and HCG in both theca and granulosa cells (Yamada *et al.* 2004). In the ovary, DEC1 appears to act as a repressor (Yamada *et al.* 2004) and either directly or indirectly alters the expression of genes in the ovary such as the follicle-stimulating hormone receptor, prostaglandin endoperoxidase synthase 2 and other E-box-dependent genes in a gonadotrophin-dependent manner. Given the expected circadian rhythm of *dec1* expression in the ovary, it is interesting to speculate that there may be circadian gating of cellular processes at the ovarian level as well as within the hypothalamus at the time of ovulation.

Oocyte and early embryo

The developing embryo can spend up to 4 days in the mouse oviduct before entering the uterus and implanting, but the impact of circadian rhythmicity during this period on embryo development is poorly understood. Presumptive maternal expression of clock genes has been described up to the two cell stage in the mouse (Hamatani 2004) and semi-quantitative measurements of clock gene expression in the embryo suggest that the expression then decreases until compaction takes place (Johnson *et al.* 2002). Neither of the studies evaluated the circadian expression of clock genes across 24 h during development and it is not clear if they are constitutively expressed at a low level as in the testis or are rhythmically expressed. Nevertheless, it is possible that the developing embryo is exposed to maternal rhythmic signals during its progress through the oviduct. We have recently shown that the rat oviduct expresses clock and clock-controlled genes rhythmically over 24 h (Kennaway *et al.* 2003*a*). Among the genes shown to be rhythmically expressed was plasminogen activator inhibitor-1 (*Pai-1*), which has been associated with the protection of the developing embryo during its transport along the oviduct (Kouba *et al.* 2000). It is likely that a systematic evaluation of gene expression profiles within the oviduct would uncover additional rhythmically secreted proteins that promote healthy and timely embryo development. The importance of this rhythmicity for the developing embryo is not known, but when an embryo is cultured *in vitro*, there are no rhythmic changes in its environment.

Uterine preparation for implantation

The uterus expresses clock genes rhythmically (Johnson *et al.* 2002, Horard *et al.* 2004, Dolatshad *et al.* 2006). A recent report investigated the effects of continuous administration of oestradiol on *per1* and *per2* expression in ovariectomised (OVX) rat uteri (Nakamura *et al.* 2005). While *per1* and *per2* mRNA levels peaked in the uteri of untreated OVX rats in the late light and early dark periods respectively, oestradiol-treated rats exhibited a biphasic expression pattern. In contrast, the treatment had no effect on the *per1* rhythm in the SCN and only slightly but significantly advanced the peak of *per2* expression. The mechanisms and physiological role of these interactions are not clear, especially since there are apparently no oestrogen response elements in the regulatory 5′-flanking regions of the *per1* and *per2* genes.

There has been only one study to date on the expression of clock-controlled genes in the uterus (Horard *et al.* 2004). The authors investigated the expression of *rev erbα* and oestrogen receptor-related receptor α (*errα*) in non-pregnant uteri and reported that both genes were expressed rhythmically with a high amplitude and peak expression in mid light and early dark respectively. While *rev erbα* is known to be induced by CLOCK/BMAL1, neither PER2, REV ERBα nor CLOCK/BMAL1 appeared to regulate *errα* mRNA expression *in vitro* (Horard *et al.* 2004). The nature of the signal responsible for the *errα* mRNA rhythm and the precise function of this protein in the uterus are not known.

We can only speculate about the role of uterine rhythmicity in embryo development, as there have been no direct studies. However, mice forced to follow non-circadian photoperiods of 22 and 26 h (which are outside the limits of entrainment) have a high level of resorption and poor pup survival (Endo & Watanabe 1989). At the time of implantation, there are changes in HIF and VEGF production, increased angiogenesis, downregulation of anti-adhesion proteins (MUC1 and MUC4) and upregulation of adhesion proteins. While the expression of these genes is affected by changes in oestrogen, progesterone and oxygen tension among others, several of these genes and proteins are known to interact with clock gene transcription factors. For example, BMAL1 can dimerise with HIF1α *in vitro* and potentially bind to hypoxia response elements in gene promoters and drive the transcription of target genes (Hogenesch *et al.* 1998). Whether the BMAL1/HIF1α dimer can induce VEGF transcription *in vivo* has been questioned (Cowden & Simon 2002). Moreover, these authors reported that *Bmal1* null embryos exhibited normal vasculature at both embryonic days 9.5 and 10.5 in contrast to that seen in *arnt* null embryos. This would seem to eliminate a proactive role of embryonic clock gene expression in the normal embryo development; however, we have preliminary evidence from our own colony of *Bmal1* null mutants that there is a significant loss of homozygous *Bmal1* null embryos in heterozygous dams during gestation resulting in skewed genotype ratios and suggesting an important role for embryonic *Bmal1* in early development (M J Boden and D J Kennaway, unpublished observations).

Reproductive consequences of genomic clock gene disruption

If circadian rhythmicity and, in particular, clock genes are important for the optimisation of fertility, then disrupting circadian rhythmicity at the gene level should result in a poor reproductive outcome. As indicated earlier in the review, circadian rhythms in behaviour and tissue gene expression have been studied in mice with null mutations through genetic manipulation as well as in chemically induced and spontaneous mutants. There have been no reports of impaired reproductive function to date in *per1*, *per2* or *per3* knockout mice either singly or in combination or in *cry1*, *cry2* and *cry1*/*cry2* double knockout mice. Indeed, the successful generation and maintenance of the knockout animals suggest that they do not have a profoundly poor reproductive phenotype, although reproductive defects can be obscured if the lines are always maintained by heterozygous matings. However, 'the phenotype of the mouse may indicate as much about the ability of specific systems to adapt to the absence of a gene product as it does about the normal role of the gene product' (Bae & Weaver 2003). Another possibility is that despite the impairment to their endogenous circadian timing system, the clock gene knockout mice are *behaviourally* entrained to the LD cycle (possibly through photophobic responses) and this may provide sufficient synchrony to allow ovulation and mating to progress, although perhaps less efficiently.

Early anecdotal evidence suggested that the $Clock^{\Delta 19}$ mutant mouse had a reproductive defect. These animals were apparently initially maintained through heterozygous mating due to infertility in the homozygote animals (Low-Zeddies & Takahashi 2001). Poor responsiveness to exogenous gonadotrophins in $Clock^{\Delta 19}$ mutants was also mentioned (without direct evidence) in one paper (Herzog *et al.* 2000), while evidence for longer oestrus cycles and poor breeding rates was provided in another study (Chappell *et al.* 2003). The first comprehensive study of reproductive function in an animal with genomic disruption of circadian rhythmicity was conducted in the $Clock^{\Delta 19}$ (C57Bl) mouse (Miller *et al.* 2004). As previously indicated, this mutant carries a 51aa deletion in the transcription activation domain of the CLOCK protein. Although the mutant $CLOCK^{\Delta 19}$ protein apparently heterodimerises with BMAL1, the complex does not induce transcription at the CACGTG E-boxes of clock or clock-controlled genes. Prolonged and irregular oestrous cycles with long periods in the oestrus phase were reported, although the timing of puberty (vaginal opening) was normal (Miller *et al.*

2004). The ovaries appeared morphologically normal with developing follicles and corpora lutea present. Thus, it could be presumed that the LH surge mechanisms were functional despite the loss of SCN rhythmicity. Curiously, however, when the blood samples were collected from *Clock*$^{\Delta 19}$ (C57BL) mutant mice from 5 h after lights on during the morning of pro-oestrus until 3 h before dawn on the morning of presumptive oestrus, none of the mutant mice showed a significant rise in LH (Miller *et al.* 2004). In contrast, 50% of the wild-type mice showed late afternoon surges in LH that lasted up to 6 h. How *Clock*$^{\Delta 19}$ mutants could ovulate in the absence of LH surge is not known, but since the authors did not provide evidence that ovulation occurred in the mutants during the course of blood sampling, one possibility is that the mutants were more susceptible to the stresses of the procedure resulting in a failure to progress to ovulation.

Clock$^{\Delta 19}$ (C57BL) mutant mice mated and fertilised ova, but by day 11 post-conception, 40% of the embryos had resorbed and a high proportion of females had an extended non-productive labour or resorption of full-term foetuses (Miller *et al.* 2004). It did appear, however, that the wild-type line also had a high level of reproductive wastage and it may be that the presence of the mutation exacerbated an existing problem in the strain. The authors also identified abnormalities in oestradiol and progesterone levels during pregnancy in the mutants as well as a shorter pseudo-pregnancy.

The presence of a homozygous *Clock*$^{\Delta 19}$ mutation in the embryo is not associated with any significant prenatal lethality, since the products of various crosses resulted in the expected ratios of genotypes and similar sex ratios (Dolatshad *et al.* 2006). These authors confirmed the longer oestrous cycles in *Clock*$^{\Delta 19}$ (C57BL) mutants and suggested that this was due to a longer period of time spent in the oestrus phase. The disrupted cyclicity was exacerbated when the mutants were kept in constant darkness and became behaviourally arrhythmic. Dolatshad *et al.* reported that the *Clock*$^{\Delta 19}$ (C57BL) mutant mice had lower fecundity than wild-type mice and in contrast to the previous report (Miller *et al.* 2004), pregnancy losses were independent of genotype in animals kept in normal lighting conditions. However, the *Clock*$^{\Delta 19}$ (C57BL) mutants had higher pregnancy losses when they were kept in continuous darkness. In agreement with the original studies, there were higher proportions of difficult deliveries in the mutants.

Homozygous *Clock*$^{\Delta 19}$ (Balb/c) mice took 2–3 days longer to mate and subsequently deliver pups compared to their wild-type control line (Kennaway *et al.* 2005). The litter size was slightly but significantly reduced (8 vs 7 pups) and survival to weaning reduced from 94 to 84%. When *Clock*$^{\Delta 19}$ (Balb/c) mice were kept in continuous darkness for 16 days, they became behaviourally arrhythmic but 7/7 animals became pregnant and delivered live offspring that had good survival to weaning (93%).

All commonly used laboratory mouse strains except the CBA and C3H strains are melatonin-deficient (Ebihara *et al.* 1986) due to mutations in pineal gland acetyltransferase (AA-NAT) (Roseboom *et al.* 1998) and hydroxyindole-*O*-methyltransferase (HIOMT). Given the central role that melatonin plays in transferring photoperiod information to the rest of the body, we considered it important to reduce any confounding impact of melatonin deficiency on reproduction in our own studies of the impact of the *Clock*$^{\Delta 19}$ mutations. Therefore, we, developed a variant of the *Clock*$^{\Delta 19}$ mouse by selective breeding of *Clock*$^{\Delta 19}$ (Balb/c) mutants with CBA mice (Kennaway *et al.* 2003*b*). This mutant strain has functional AA-NAT and HIOMT enzymes and hence is able to synthesise melatonin. Studies indicated that the *Clock*$^{\Delta 19}$+MEL mouse actually maintains a rhythm of melatonin production and secretion in a 12 h light:12 h darkness photoperiod with peak secretion occurring 2–3 h later than the wild-type mice (just prior to lights on) (Kennaway *et al.* 2003*b*). In contrast to this maintenance of central rhythmicity, the *Clock*$^{\Delta 19}$+MEL mutants have peripheral tissue arrhythmicity (Kennaway *et al.* 2006).

In *Clock*$^{\Delta 19}$+MEL mice, there was a significant small delay in the time to achieve pregnancy, reduced survival to weaning (80 vs 96% in wild type animals), but a significant small increase in litter size. Mating of *Clock*$^{\Delta 19}$+MEL mice in constant darkness was successful even in those mice that exhibited behavioural arrhythmicity; however, as in the case of this strain maintained in LD cycle, survival to weaning was poor (Kennaway *et al.* 2005). Finally, we found that despite an apparently normal ovulation rate and litter size, immature female *Clock*$^{\Delta 19}$+MEL mice treated with PMSG/HCG had a lower percentage of successful matings and fewer embryos were recovered 96 h post-HCG injection (Kennaway *et al.* 2005).

In summary, the *Clock*$^{\Delta 19}$ mutation has significant but subtle effects on reproductive function, which reduce fertility and fecundity without manifesting an obvious infertility. There is a need to investigate the mutants further to answer some of the puzzling aspects of their reproductive function, in particular the apparent lack of a normal pro-oestrus and exogenous oestradiol-induced LH surge in *Clock*$^{\Delta 19}$ (C57Bl) mice (Miller *et al.* 2004). Dolatshad *et al.* raised the possibility that the mating act may cause the LH surge and subsequent ovulation in the *Clock*$^{\Delta 19}$ mutants (Dolatshad *et al.* 2006). They also suggested that increased leptin could be involved at a more direct ovarian level (Barkan *et al.* 2005), a possibility that is strengthened by the report that the *Clock*$^{\Delta 19}$ (C57Bl) mutant is hyperleptinemic (Turek *et al.* 2005). It is important to keep in mind the effects of the background strain on the severity of the reproductive defects caused by the *Clock*$^{\Delta 19}$ mutation.

The *Bmal1* knockout mouse (Bunger *et al.* 2000) has been shown to have profoundly disrupted behavioural rhythmicity and loss of gene rhythmicity in the SCN and peripheral tissues in anormal LD cycle. In the original description of the production of this null mutant, no mention was made of the fertility of the homozygous females. Indeed, *Bmal1* mutant mice have been described as 'viable and fertile' (Cowden & Simon 2002). Further, it has been stated that 'matings of mice heterozygous for the *Mop3* (*Bmal1*) null allele resulted in births of each expected genotype with a ratio that was consistent with a locus that is not essential for normal embryonic development' (Bunger *et al.* 2005). Preliminary studies in our laboratory, however, have uncovered impaired fertility in both males and females, including delayed puberty, smaller ovaries and uteri and an inability to establish viable pregnancies (Boden & Kennaway 2004, 2005). Moreover, in our colony, the ratio of genotypes following heterozygous mating is skewed with fewer homozygous null *Bmal1* offspring produced than expected, suggesting a degree of embryo lethality in the *Bmal1* null mice. Thus, in keeping with its major role in initiating cellular rhythmicity, this profound infertility is the strongest evidence yet for the role of clock genes in reproduction.

The Tau hamster, which has a mutated casein kinase 1ε, has a mildly perturbed reproductive profile. Ovariectomised (OVX) Tau mutant hamsters show a lower LH pulse frequency than OVX wild-type hamsters (Loudon *et al.* 1994). Further, the timing of circadian behaviour patterns is synchronised with the circadian hormone oscillations of melatonin, cortisol and LH (Lucas *et al.* 1999), such that the timing of the LH surge and hence (in non-OVX animals) ovulation remains co-ordinated to the SCN, even in animals maintaining a non-24 h rhythm. There is no documented difference in sexual performance in the female Tau hamster (Refinetti & Menaker 1992), while male Tau hamsters maintain reproductive seasonality. However, this seasonality is linked to their endogenous period length (20 h), so that a light duration in excess of 10 h is sufficient to drive seasonal fertility, whereas the wild-type hamster requires >12 h light per day (Stirland *et al.* 1996). This observation confirms the strong link between environmental light and the internal timing system of the SCN.

Circadian rhythm disruption in humans

There are no reports to date of any associations between clock gene mutations and reproductive function in humans. However, polymorphisms in clock genes have been discovered in humans, which result in subtle changes in behavioural (sleep/wake) rhythms similar to those found in the animal models and these may be worth investigating in the future. For example, a family shown to have a mutation in *per2* (S662G) that prevents the phosphorylation cascade of the PER2 protein by casein kinase 1ε have advanced sleep phase syndrome (ASPS; i.e. they have early sleep onset and earlier waking times). By reducing PER2 phosphorylation and extending its half life, the endogenous cellular rhythmicity proceeds faster, resulting in a 4 h earlier appearance of sleep, melatonin rhythm and other circadian markers (Toh *et al.* 2001) similar to that found in the Tau hamster.

A mutation in casein kinase 1ε gene (S408N) has been identified (Takano *et al.* 2004), which results in the loss of serine in the enzyme. Since phosphorylation of this serine normally reduces the enzyme activity, the mutation increased the activity of the enzyme *in vitro* 1.8-fold (whereas the loss of all eight sites increased enzyme activity by eightfold; Gietzen & Virshup 1999). Subjects carrying the S408N polymorphism were less likely to suffer from delayed sleep phase syndrome (DSPS) or non-24 h sleep/wake syndrome compared to a normal population. A more active casein kinase 1ε leading to a protection from DSPS (i.e. presumptive long endogenous period) seems a contradiction in light of the phenotype of the Tau hamster, where reduced casein kinase 1ε activity has associated with the equivalent of ASPS. However, it has been suggested that this response could be due to changes in PER3 phosphorylation, as the inability to phosphorylate PER3 in humans is correlated with an increased risk of DSPS (Ebisawa *et al.* 2001, Archer *et al.* 2003).

Polymorphisms in the human *Clock* gene (in particular, the T3111C allele) have been documented, but the exact physiological importance of these is still under debate, with some evidence associated with DSPS (between 10 and 44 min delay per day) (Katzenberg *et al.* 1998), particularly in patients suffering from depression (Iwase *et al.* 2002) as well as insomnia in bipolar depression patients (Benedetti *et al.* 2003, Serretti *et al.* 2003). Other research has suggested that the T3111C polymorphism is associated with DSPS (Mishima *et al.* 2005), while others have failed to find a correlation (Robilliard *et al.* 2002).

Period gene polymorphisms have been investigated in breast tissue, in relation to a potential link with breast cancer (Chen *et al.* 2005). The results from this study showed that one or more PER proteins were expressed differently in cancer cells compared to normal tissues in 96% of the patients studied. While several polymorphisms in the *per* genes were identified in this study, the methylation status of the promoters had the greatest impact on PER protein expression in the breast cells. Mammary tissue gene expression has been shown to be rhythmic in the mouse (Metz *et al.* 2006) and *per2* has been suggested to have a role in tumour development and anti-cancer function, with *per2* knockout mice being

more prone to developing cancer and less resistant to radiation-induced cancer formation (Fu *et al.* 2002).

Shift work and fertility

There is a growing trend for people to work outside normal daylight hours. This alters the timing of exposure to light and potentially alters SCN and peripheral tissue rhythmicity. It might be expected that this could in turn impact on the physiological systems influenced by the circadian timing system, including reproduction. Shift workers do not always adjust their circadian rhythms (e.g. melatonin) appropriately to their imposed shift schedule (Dumont *et al.* 2001). Women shift workers and airline flight attendants have been reported to have an increased risk of spontaneous abortions, irregular menstrual cycles, low birth weight babies and an increased incidence of premature birth (Bisanti *et al.* 1996, Knutsson 2003). In other studies, however, the association was reported only as a trend that increased as the amount of hours worked per month increased, (Cone *et al.* 1998, Aspholm *et al.* 1999) or not found at all (Zhu *et al.* 2003), perhaps suggesting that a strong and persistent circadian disruption may be needed for an impact on fertility in women. Labyak *et al.* reported that half the shift working nurses surveyed experienced changes in menstrual function when working shift work (Labyak *et al.* 2002). Furthermore, 3 of the 68 nurses, working in shift work, reported infertility, while they were attempting to conceive. More studies are required to determine whether rhythm disturbance associated with shift work does impact on female fertility.

Conclusion

In conclusion, there is growing evidence that circadian rhythmicity and reproduction are interconnected. The circadian timing system influences a wide range of physiological systems via hormonal and neural routes and the disruption of clock gene expression can lead to a spectrum of reproductive (Miller *et al.* 2004) and metabolic changes in animal models (Turek *et al.* 2005). As with any knockout models where the defect is present throughout life, the maintenance of fertility in many clock gene mutants points to a high level of redundancy in the circadian timing system. An exception may be the *Bmal1* gene, which appears to be crucial and when the function of this gene is disrupted, there are severe alterations to reproductive function. A role for the circadian timing system in human reproduction has neither been definitely established, nor systematically studied. Polymorphisms in human clock genes have been identified and are associated with alterations in the timing of sleep–wake cycles and so it is perhaps to be expected that some of these polymorphisms will be found to influence human fertility. The influence of rhythmic gene expression in the embryo remains to be addressed, while possible links between circadian rhythm dysfunction, metabolic syndrome and fertility are also unexplored. In the next few years, it is likely that these links will become clearer as an appreciation of the profound rhythmicity of the transcriptome grows.

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