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The role of circadian rhythmicity in reproduction

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Circadian rhythmicity is evident in a wide range of physiological systems including the reproductive axis. The recent discoveries of rhythmic clock gene expression in peripheral tissues, including reproductive tissue, suggests that they may play an important role in optimizing fertility. The evidence for rhythmic control of reproduction from studies in laboratory animals is reviewed and where possible this includes evidence from human studies. Clock genes are highly conserved across species including humans and there is no reason to suggest that they are functionless in humans. The challenge issued here is for researchers to probe their function and the consequences of their disruption in both animal and human reproduction.

Key words: clock/clock genes/period/shiftwork/suprachiasmatic

Introduction

In recent years our understanding of the origins, control and importance of biological rhythmicity has increased tremendously. In 1997, 1998 and 2002 for example the journal *Science* recognized advances in the field in its 'Breakthrough of the Year' lists. In 2003 the journal *Nature* published an Editorial entitled 'Timing is everything' (Anonymous, 2003), again highlighting the progress in our understanding of daily circadian rhythmicity and its role in normal physiological functions and in certain diseases. The aim of this review is to provide an update of our understanding of the relationship between circadian rhythms and reproductive processes.

Circadian rhythms

Before embarking on a review of rhythms and reproduction it is important to first establish some of the terminology and background in chronobiological research. A circadian rhythm is a biological rhythm that persists under constant environmental conditions (light, dark, temperature) with a period length of ~24 h. Its phase can be reset by a brief interruption in the constant regimen. The persistence of rhythmicity in a constant environment such as continuous darkness implies that the rhythm is generated endogenously rather than in reaction to the external environment. The capacity for resetting is important in the context of seasonal adjustment to the duration of the day-length and becomes profoundly important for humans endeavouring to work and sleep outside normal working hours or travelling through multiple time zones.

The circadian timing system consists of an input pathway connecting the clock to the environment, the clock itself and output pathways. The site of the 'master biological clock' in mammals is the suprachiasmatic nucleus (SCN), which is a paired concentration of small neurons and glial cells in the anterior hypothalamus directly above the optic chiasm and divided by the third ventricle. The SCN cells express an endogenous precise rhythmicity of function that in most species is different from 24 h (as demonstrated by the periodicity of various systems in constant darkness (Middleton *et al.*, 1996), following surgical isolation of the SCN *in vivo* (Inouye and Kawamura, 1979) and in SCN organ culture experiments *in vitro* (Green and Gillette, 1982) and so must be reset each day. Entrainment is achieved directly via neural input from the eyes via the optic tract (retino-hypothalamic tract), as well as indirectly via input from the intergeniculate leaflet, raphe nuclei and some other lesser important centres (Morin, 1994). The SCN cells contain a wide range of neurotransmitters, hormones and cytokines as well as receptors for an equally wide range of ligands (Table I). An obvious consequence of the localization of the neurotransmitter and hormone receptors in the SCN is that the biological clock and circadian rhythmicity can potentially be altered by drugs and endogenous hormones.

The output component of the circadian timing system translates internal time into physiological action. The SCN achieves this directly by secreted factor(s) and via multisynaptic neural pathways. The SCN has long been known to secrete material into the cerebrospinal fluid (CSF) that influences behaviour since circadian behavioural rhythms can be re-established in

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Table I. A short tabulation of some of the transmitters, receptors and hormones that have been identified in the suprachiasmatic nucleus

Neurotransmitter	Neurotransmitter receptors	Hormone and cytokine receptors
PACAP (pituitary adenylate cyclase-activating polypeptide.)	Serotonin (1A, 1B, 2C, 5, 7)	Melatonin
γ-Aminobutyric acid	*N*-Methyl-D-aspartate	Estrogen
Vasoactive intestinal peptide	Neurotensin	Insulin-like growth factor-I
Serotonin	NGF (Nerve growth factor)	Leptin
Glutamate	Vasopressin (V1A)	Interferon
Neuropeptide Y	Somatostatin	
	Cholinergic (muscarinic and nicotinic VPAC2 (Vasoactive intestinal peptide receptor type 2)	

arrhythmic SCN-lesioned animals by transplantation of SCN tissue into the third ventricle (Silver *et al.*, 1996). Secreted factor(s), not new neuronal connections, are responsible for the re-establishment of rhythmicity. Importantly not all rhythms were re-established and the rhythms were not entrained by light/dark. It is now known that vasopressin (AVP) (Schwartz and Reppert, 1985), transforming growth factor (TGFα) (Kramer *et al.*, 2001) and prokineticin 2 (PK2) (Cheng *et al.*, 2002) are secreted into the CSF. AVP is likely to be involved in the temperature rhythmicity; of the other two peptides, PK2 has the most potent effects on wheel-running behaviour and thus potentially on sleep/wakefulness (Cheng *et al.*, 2002).

Neural pathways are the major means of controlling brain and peripheral tissue rhythmicity. The best known pathway controls the pineal gland production and secretion of melatonin. The SCN efferent neurons project to the paraventricular nuclei (PVN) which in turn send efferents to the intermediolateral column of the spinal cord and on to the superior cervical ganglia. Noradrenergic neurons of the nervi conari return to the brain and synapse on pinealocytes (Larsen *et al.*, 1998; Teclemariam Mesbah *et al.*, 1999). Contrary to early suggestions that SCN lesions lead to the abolition of production and rhythmicity of melatonin, it is now thought that SCN neural output tonically suppresses melatonin production during the day, since small carefully placed SCN lesions result in continuous arrhythmic melatonin production (Kalsbeek, Garidou *et al.*, 2000a; Perreau-Lenz *et al.*, 2003).

The timing of the melatonin rhythm and changes in amplitude of the rhythm are the result of SCN activity and as such secreted melatonin provides an additional link between the circadian timing system and peripheral organs and orchestrating seasonal changes in fertility (Lincoln *et al.*, 2003b). Melatonin receptors are predominantly expressed in the SCN (Vanecek *et al.*, 1987; Reppert *et al.*, 1994) and the pars tuberalis (Williams and Morgan, 1988; Barrett *et al.*, 1996). In the former case the hormone may provide a stabilizing feedback signal to the clock (Redman *et al.*, 1983), whereas in the latter melatonin participates in the seasonal production and secretion of prolactin (Lincoln *et al.*, 2003a).

The SCN projects to other parts of the brain involved in reproduction including the medial pre-optic area (MPOA) (Palm *et al.*, 1999) and GnRH-positive cells (van der Beek *et al.*, 1997b). Studies from the Buijs laboratory have suggested that SCN vasopressin release may play a role in GnRH secretion and consequently the timing and magnitude of the LH surge (Palm *et al.*, 2001). Recent tract tracing studies have highlighted multisynaptic connections to the parasympathetic and sympathetic nervous system, resulting in SCN influences on adipocytes, heart, liver etc. (Kalsbeek, Fliers *et al.*, 2000b; la Fleur *et al.*, 2000; Buijs and Kalsbeek, 2001; Buijs *et al.*, 2001; Scheer *et al.*, 2001). It is reasonable to expect that the sympathetic innervation of the ovary and uterus may also be influenced by the SCN, although to our knowledge no such link has yet been investigated.

Molecular organization

As indicated previously, a feature of SCN rhythmicity is its endogenous nature with the near 24 h rhythmicity generated within cells of the nucleus. In the last few years the origin of this rhythmicity has been shown to be derived from gene transcription/translation feedback/feedforward loops (Reppert and Weaver, 2002). To date there are ≥10 genes that form the basis of cellular rhythmicity including *per1*, *per2*, *per3*, *Clock*, *Bmal1*, *cry1*, *cry2*, *dec1*, *dec2* and *Rev erb a* (Table II).

The key genes appear to be *Bmal1* and *Clock* since their protein products form a heterodimer and bind to a specific gene sequence, CACGTG (called an enhancer or 'E-box') in the promoter region of *per1*, *per2*, *cry1* and *cry2* genes. There is some emerging evidence that aside from the E-Box itself, the context of the enhancer motif in the promoter may be important for circadian gene induction (Munoz *et al.*, 2002). CLOCK/BMAL1 dimers drive the transcription of the period and cryptochrome genes resulting in a build up of the proteins in the cytoplasm. In the cytoplasm, phosphorylation by casein kinase 1E alters the stability of the period proteins and so limits the amount of PER protein available for dimerization with CRY and subsequent transport back into the nucleus. The entire PER1/PER2/CRY1/CRY2 complex is involved in displacing or inhibiting the transcription activity of the BMAL1/CLOCK dimer at the E-box and thus inhibiting the transcription of period and cryptochrome

Table II. Genes shown to play a role in the generation of cellular rhythmicity in vertebrates

Gene name	*Abbreviation*	Alternate name	Homologue
Period 1	*per1*		
Period 2	*per2*		
Period 3	*per3*		
Circadian Locomotor Output Cycle Kaput	*Clock*		*npas2* (*MOP4*)
Brain Muscle ARNT-Like protein 1	*Bmal1*	*MOP3*	*Bmal2*
Cryptochrome 1	*cry1*		
Cryptochrome 2	*cry2*		
Differentiated Embryo Chondrocytes 1	*dec1*	*Stra13*, *Sharp2*, *BHLHB2*, *Clast5*	
Differentiated Embryo Chondrocytes 2	*dec2*	*Sharp1*, *BHLHB3*	
Rev Erb alpha	*Rev erba*		

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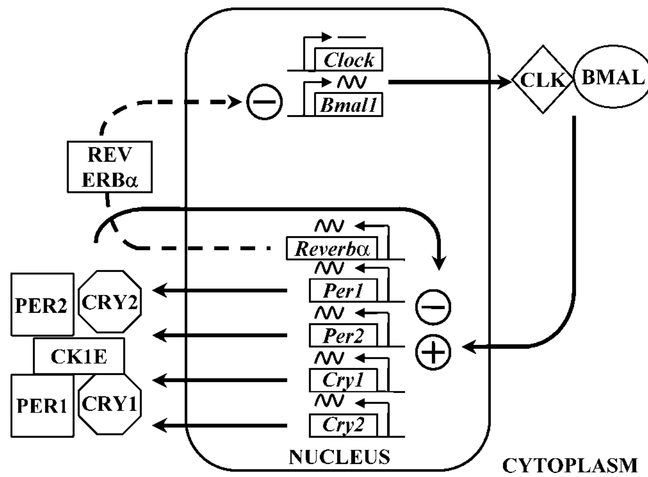

Figure 1. Schematic of the primary loops of clock gene transcription factor rhythms. Positive drive is afforded by the CLK/BMAL1 heterodimer complex which initiates transcription of *per1*, *per2*, *cry1* and *cry2*. Proteins from these genes in complex with casein kinase 1E inhibit CLK/BMAL1 induction. Meanwhile, REV ERBα protein inhibits *Bmal1* transcription. *Clock* expression is normally constitutive while *Bmal1* is rhythmic and in antiphase to the period and cryptochrome gene expression. A secondary loop not shown here involves *dec1* and *dec2* (Honma and Honma, 2003).

genes (Figure 1). This cycle takes ~24 h to complete. The critical role of the casein kinase(s) in determining the period of the cycle was demonstrated in hamsters and humans through studies on naturally occurring mutations (Lowrey *et al.*, 2000; Toh *et al.*, 2001). In the *tau* mutant hamster the unusually short circadian period of these animals (22 h) was shown to be due to a mutation in casein kinase 1E which resulted in hypophosphorylation of PER proteins (Lowrey *et al.*, 2000). With more protein available, the PER/CRY complex presumably was then able to suppress period and cryptochrome gene transcription earlier. In humans exhibiting Familial Advanced Sleep Phase Syndrome (ASPS), affected subjects were shown to have a mutation of *per2* such that a phosphorylation cascade of the protein by casein kinase was inhibited (Toh *et al.*, 2001). This again presumably resulted in a reduced rate of PER2 degradation and faster cycling of the feedback loop and an accompanying shorter circadian period and earlier onset/offset of physiological rhythms that included sleep and melatonin production.

The positive drive to the molecular rhythmicity in cells has been shown to involve PER2 induction of *Bmal1* gene expression through an unknown site (Shearman *et al.*, 2000a). To prevent this feed forward cycle from proceeding out of control, REV ERB alpha protein provides an inhibitory brake on *Bmal1* transcription (Preitner *et al.*, 2002). Further control over the cellular rhythmicity is afforded by another two genes, *dec1* and *dec2* (Honma *et al.*, 2002; Kawamoto *et al.*, 2004). Both *dec1* and *dec2* possess CACGTG E-boxes in their promoter regions and so are induced by CLOCK/BMAL1. As well as inhibiting their own transcription by interacting with CLOCK/-BMAL1, DEC1 and DEC2 also repress *per* and *cry* transcription. Taken together these clock genes and their proteins provide an intricate molecular network that establishes the intracellular timing system.

The premier position of the SCN in the circadian timing system has been dogma until the era of clock genes commenced. In the first report of a mammalian *per* gene (Tei *et al.*, 1997), it was clear that the gene was expressed widely (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis). Subsequently all of the other clock genes were shown to be expressed in a wide range of peripheral tissues, e.g. *per2* (Sakamoto *et al.*, 1999) and *Bmal1* (Oishi *et al.*, 1998). Indeed the expression of the clock genes is rhythmic and often of very high amplitude in peripheral tissues. Interestingly the phasing of the organ rhythms is often out of phase with the SCN rhythm (Yamazaki *et al.*, 2000) although the phasing suggests that the rhythmicity is driven by the SCN. When peripheral tissues (liver, muscle and lung) were maintained in culture, luciferase transgene activity driven by a *per1* promoter was rhythmic for only a few cycles before damping out, in contrast to the SCN in which rhythmic activity continued for >30 cycles. A recent study has, however, prompted a reassessment of the concept of a strict hierarchical control of rhythms by the SCN. Using a different *per2*:luciferase reporter, self-sustained circadian oscillations were maintained *in vitro* in liver, pituitary, kidney, lung and cornea for >20 cycles (Yoo *et al.*, 2004). Lesion experiments confirmed that the circadian rhythms in the peripheral tissues did not depend on the SCN. The SCN maintains its 'master' role and plays a major role in the entrainment of the molecular timing of organ systems, but under certain circumstances, other influences such as stress (Balsalobre *et al.*, 2000a) and food availability (Damiola *et al.*, 2000) appear able to modify the timing/phasing of the tissue rhythmicity.

The demonstration of endogenous circadian rhythmicity in peripheral tissues is extremely important because several of the clock gene transcription factors form part of an output pathway. Microarray analysis of liver, heart, kidney, muscle as well as the SCN have shown that between 100 and 400 genes are rhythmically expressed (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002; Oishi *et al.*, 2003). Moreover, not all tissues express the same genes rhythmically although there is an overlap of a core set of ~20–40 genes (Delaunay and Laudet, 2002). This astonishing situation stems from the fact that many transcription factors (e.g. *dbp*, *usf2*, *myc*, *max*, *zfp36*, *sox3*, *tef* and *arnt2*) and functional genes (e.g. vasopressin, *pai-1*) possess E-box motifs in their promoters that are activated by the cyclic appearance of the CLOCK/BMAL1 heterodimer complex (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002; Oishi *et al.*, 2003). DEC1 which has a role in rhythm generation is also known to be a potent repressor of genes (Grechez-Cassiau *et al.*, 2004) as well as being itself induced by insulin (Yamada *et al.*, 2003) and gonadotrophins (Yamada *et al.*, 2004). The impact of these findings on the molecular biology of reproductive events is likely to be huge, but the field is only in its early stages. At the very least, it should no longer be considered acceptable to make or report measurements of gene expression in reproductive tissue without reporting the time of day the tissue was collected; preferably multiple time-points should be analysed to rule out the possibility of major circadian swings in activity and function.

As indicated previously, an important characteristic of circadian rhythmicity is the ability of rhythms to be entrained to the environment, but this aspect of molecular chronobiology is not completely understood. The most likely process is through induction of one or more of the core clock genes, e.g. the *per* or *cry* genes via a non-E-box site. A prime candidate for this

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re-setting is *per1* since it appears to be easily induced by light in the SCN *in vivo* (Shigeyoshi *et al.*, 1997) and by various inducers in cells maintained *in vitro*, including calcium and glucocorticoids (Balsalobre *et al.*, 2000a,b), serum (Balsalobre *et al.*, 1998), forskolin (Yagita and Okamura, 2000), 4β-phorbol-12-myristate-13-acetate (PMA) (Motzkus *et al.*, 2000) and interleukin-6 (Motzkus *et al.*, 2002).

Rhythms and reproduction

Circadian rhythmicity of physiological systems can have several purposes in reproduction. The most obvious is to provide an organism with the sense of time of day to ensure that physiological and behavioural events coincide. An example would be the coordination of ovulation, receptivity and wakefulness in the female with activity/wakefulness in the male. In many species, especially those with short life cycles or a duration of gestation and time to weaning of <1 year, circadian rhythms can provide a sense of time of year. Thus the changes in the time of the re-entraining stimulus of light may be associated with tightly controlled seasonal onset of puberty and adult infertility/fertility cycles.

Puberty

Since puberty reflects the maturation of all systems required for optimal reproductive performance it may be expected that circadian rhythmicity will have a role in puberty. In rodents, puberty onset is altered by daylength such that long durations of darkness inhibit sexual maturation (Reiter, 1980). The impact of short daylength is obvious in animals such as hamsters, which, outside the laboratory setting, live in higher latitudes and/or environments where food availability is highly seasonal. In longer-lived species such as sheep, the time of puberty of animals born late in the season may even be delayed until the following year (Foster *et al.*, 1988). The advantages to a species of restricting onset of fertility and the resulting pregnancy and offspring care to favourable times of the year are obvious. In the case of laboratory rats and mice maintained for hundreds of generations in constant environmental conditions and selected for high fertility and fecundity, circadian or photoperiodic effects on puberty are far less common (Clark and Price, 1981; Lee and McClintock, 1986). Nevertheless some strains, e.g. Fisher 344 rats, do retain photo-responsiveness, although the basis for this is not known (Leadem, 1988). Some clues to the residual photosensitivity may lie in the well-known observation that some rat strains become responsive to short daylength when they are made anosmic (Blask and Nodelman, 1980) or fed a protein-restricted diet (Blask *et al.*, 1980). It may be that these perturbations reinstate sensitivity to hormonal rhythms (e.g. melatonin) that have been selected against in animal breeding facilities. Note also that of the commonly used laboratory mouse strains, only CBA and C3H mice have functional AA-NAT and HIOMT enzymes that allow them to synthesize melatonin and so translate photoperiod information into a hormonal signal (Ebihara *et al.*, 1986; Goto *et al.*, 1989). As previously indicated there is new evidence that periodic food restriction in laboratory rodents can entrain circadian rhythms in gene expression (Damiola *et al.*, 2000) and so a clue to photo-responsiveness may lie in future studies of clock gene expression in peripheral tissues of photo-responsive and non-responsive strains.

Human puberty

Studies on the seasonal occurrence of menarche in humans that are often reported (Bronson, 1995) are difficult to interpret in circadian or photoperiod terms since it is strongly influenced by size at birth (Adair, 2001) nutrition, climate, stress and living conditions (Wolanski *et al.*, 1994; Gueresi, 1997). Nevertheless it has long been known that during puberty there is the appearance of remarkable rhythmicity in gonadotrophin secretion and steroid secretion in boys and girls (Boyar *et al.*, 1972). Highest hormone levels are observed during the night, but this rhythmicity disappears in adult life. It is interesting, given the above discussion of nutritional sensitization to daylength in rodents, that hormonal rhythmicity often returns in women with anorexia nervosa. The return of menstrual function, however, does not show a simple relationship to weight, fatness, or maturity of LH pattern (Katz *et al.*, 1978). It is worth noting that the early report of puberty in humans coinciding with a precipitous decrease in rhythmic melatonin production (Silman *et al.*, 1979) has not been confirmed and in fact melatonin production is remarkably constant during puberty (Tetsuo *et al.*, 1982).

Adult reproductive function

Males

There are strong seasonal rhythms in reproductive function in males of many species, best characterized in hamsters (Reiter, 1980) and sheep (Lincoln, 2003). Thus rams are able to decode the daily changes in light across the year and to translate these into hormonal signals that reduce and then reinitiate gonadal function and libido. During the sexual season, however, there is little evidence of circadian rhythmicity in hormone secretion, apart from prolactin and melatonin (Kennaway *et al.*, 1981).

Several groups have investigated clock gene expression in the testes of laboratory animals. Remarkably the testis appears to be the only tissue studied to date that does not exhibit overt rhythmicity of clock gene expression. Morse *et al.* (2003) examined clock gene expression in the testes of mice on the premise that the developmental events occurring in spermatogonia, spermocytes and spermatids appear to be coordinated. They hypothesized that biological clocks might influence developmental decisions taken by each of the different cell types. They found that *per1* was expressed in mouse tissue but there was no apparent change in expression across 24 h. Given the major role of *Bmal1* in the control of rhythmic *per1* expression, it was not surprising that *Bmal1* expression was also non-rhythmic in the testis. PER1 protein was also constantly present across 24 h. Further analysis indicated that *per1* expression was highest during stages VII–X of sperm development, suggesting that the expression may be developmentally regulated. Testis *per1* expression was maintained in *Clock* mutant mice, suggesting that an alternate control pathway exists for testicular *per1* expression compared to other peripheral tissues. These results were subsequently confirmed and *per2*, *cry1* and *npas2* added to the list of clock genes expressed in developing sperm and Leydig cells non-rhythmically (Alvarez *et al.*, 2003; Bittman

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et al., 2003). Clock gene expression in Syrian hamster testis has also been investigated (Tong *et al.*, 2004) and *per1* transcription (in this case both a long and short transcript) peaked late in the subjective night. By contrast, rhythmicity of *Bmal1* expression in long photoperiods was equivocal, while neither *per2* and *Clock* expression showed significant variation with the time of day. The authors also showed in this photoperiodic species that long-term exposure to continuous darkness to produce gonadal regression altered the pattern of clock gene expression in the testis. Under these conditions, expression of the *per1* transcripts became arrhythmic while *Bmal1* expression was profoundly rhythmic. Thus although there is seasonality of male reproduction in many species, it would appear that if circadian rhythmicity at the gonadal level drives these changes it involves a different rhythm-generating mechanism.

There have not been any systematic studies of reproductive function in male mice with disrupted clock genes. All clock gene mutant and knockout mice are maintained as homozygous colonies and there have been no reports or comments implying impaired fertility in the males (Vitaterna *et al.*, 1994; Thresher *et al.*, 1998; van der Horst *et al.*, 1999; Zheng *et al.*, 1999, 2001; Bunger *et al.*, 2000; Shearman *et al.*, 2000b; Bae *et al.*, 2001). One important knockout mouse, the *Bmal1* null, deserves mention, however. In the paper describing the generation of this null mutant (Bunger *et al.*, 2000), no mention was made of the fertility of the males or females; however, on establishing our own colony of *Bmal1* knockout mice from the Bradfield laboratory we were advised that they must be maintained as heterozygotes due to impaired fertility. This has been confirmed in our colony and we have gone on to show that adult males have reduced seminal vesicle weight, normal testis weight and a very high incidence of abnormal sperm (M.J.Boden and D.J.Kennaway, unpublished data). Homozygous *Bmal1* knockouts are rarely able (one out of 15 males in one study) to successfully fertilize normal females. The basis for this defect is not known, but is currently under investigation.

Females

Circadian rhythms are known to play a critical role in the estrous cycle of laboratory rodents. Rats and mice initiate their LH surge in the late afternoon of pro-estrus, with subsequent ovulation and mating occurring ~6 h after darkness. There have been several excellent reviews on this topic (Turek *et al.*, 1984; van der Beek, 1996; Barbacka-Surowiak *et al.*, 2003), but a number of features need to be reiterated here. Lesions of the SCN block ovulation and abolish the LH surge in intact and ovariectomized steroid-treated animals. Administration of pentobarbital prior to pro-estrus acutely inhibits the LH surge and ovulation, but remarkably both events occur 24 h later at the expected time of day. The timing of the ovulatory process is clearly determined by the SCN.

The SCN is a target for gonadal steroids, is sexually dimorphic and the steroid milieu during development may influence SCN neurons, determining in part gender-specific rhythmicity (Swaab *et al.*, 1995). An example of the effects of gender on rhythmicity is the change in wheel-running activity onset that occurs at estrus in laboratory rodents brought about by the elevated estrogen levels (Albers *et al.*, 1981; Wollnik and Turek, 1988). Thus on the evening of pro-estrus the animals commence their circadian activity earlier than on other days of the estrous cycle. It is not known how the steroids affect SCN function, but one suggestion is that estradiol enhances the expression of immediate early gene and transcription factor responses to light in SCN cells (Abizaid *et al.*, 2004) through estrogen receptor-positive neurons projecting to the SCN (de la Iglesia *et al.*, 1999). Recent studies have confirmed the presence of estrogen receptors in the SCN (Shughrue *et al.*, 1997; Su *et al.*, 2001; Shima *et al.*, 2003).

For the SCN to influence the LH surge and ovulation, it presumably affects hypothalamic production and/or secretion of GnRH. Connectivity with estrogen-sensitive medial preoptic area neurons (Watson *et al.*, 1995) and with GnRH-containing neurons has been described (van der Beek *et al.*, 1993, 1997a; de la Iglesia *et al.*, 1995). Previously in this review it was mentioned that clock gene rhythmicity occurred in cells and tissues not traditionally recognized as exhibiting circadian rhythmicity. Rhythmic *per1* and *per2* gene expression has been reported in an immortalized hypothalamic GnRH-producing cell line (GT1-7) following synchronization with serum (Gillespie *et al.*, 2003) or forskolin (Chappell *et al.*, 2003) as had been previously reported for fibroblast cells. When these cells were transfected with a mutated clock gene, *Clock* $^{\Delta 19}$, the *per1* rhythm was blunted (Chappell *et al.*, 2003). Furthermore GnRH pulsatility was significantly reduced, but not absent, in the *ClockD* ^{19}-transfected cells. These results provide one of the first links between circadian clock gene expression and ultradian hormone pulses, although they do not provide clues to any of the underlying mechanisms. In summary, SCN rhythmicity ensures optimal temporal integration of behaviour, ovulation, fertilization and embryo development (Figure 2).

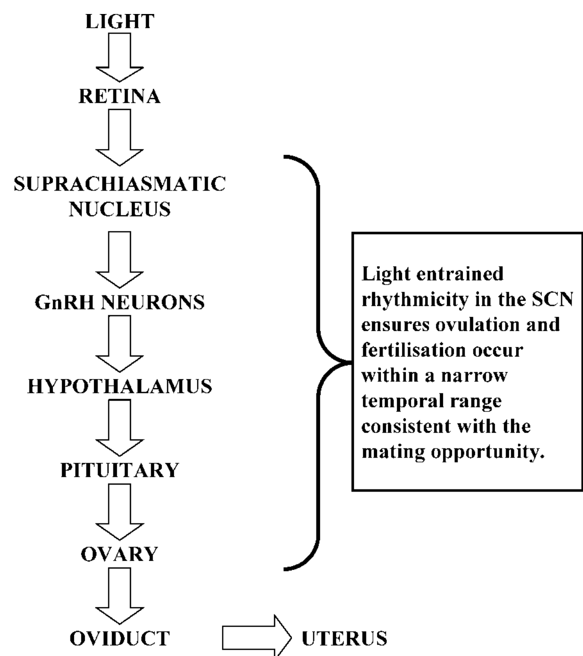

Figure 2. Schematic of the link between the environment (light/dark) and the female reproductive tract. Rhythmicity at each level ensures optimal embryo development.

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Clock gene disruption and female fertility

All known clock genes have been disrupted using conventional knockout techniques (Thresher *et al.*, 1998; van der Horst *et al.*, 1999; Zheng *et al.*, 1999; Bunger *et al.*, 2000; Shearman *et al.*, 2000b; Bae *et al.*, 2001; Zheng *et al.*, 2001) except *Clock* and in most of these cases no protein is produced. In the case of *Clock*, a mutant has been produced through *in vivo N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (Vitaterna *et al.*, 1994). In the *Clock* $^{\Delta 19}$ mutant mouse, an A → T transversion in the splice donor site downstream from exon 19 leads to the skipping of this exon in the *Clock* mRNA and elimination of 51 amino acids in the C-terminal glutamine-rich region of the CLOCK protein (King *et al.*, 1997). The BMAL1/CLOCKD^{19} heterodimer lacks the ability to initiate transcription at an E-box (Gekakis *et al.*, 1998). Even in those mutants that are arrhythmic in constant darkness such as *cry1*/*cry2* double knockouts (van der Horst *et al.*, 1999) there are apparently no major effects on fertility. By way of caution, however, it must be acknowledged that there have been no published systematic studies of fertility in these knockouts and so small changes and/or compensation may have been missed.

There have been some fertility concerns raised about the *Clock* mutant mouse. Mice with this mutation have been described as having altered fertility in that they could not be maintained through homozygous pairing due to 'an as yet uncharacterized parturition defect' (Low-Zeddies and Takahashi, 2001) although this was said to no longer be the case. There is also an anecdotal report of reduced fecundity and lack of response to ovulation induction in *Clock* mutant mice (Herzog *et al.*, 2000). A 'reduced breeding success rate' in *Clock* mutant mice was reported (Chappell *et al.*, 2003), but again there was insufficient experimental detail and very small numbers of pairings ($n = 4$). These authors did, however, provide convincing data indicating that *Clock* mutant mice exhibit prolonged estrous cycles, spending a greater length of time in estrus than wild type or heterozygous mutant animals. The longer estrus cycle length apparently persisted in constant darkness.

The first extensive evaluation of reproduction in *Clock* mutant females (Miller *et al.*, 2004) reported normal timing of puberty (vaginal opening) and confirmed the prolonged and irregular estrous cycles in these mutants. Despite apparently normal ovarian morphology and steroid levels on the afternoon of pro-estrus, *Clock* mutants apparently never exhibited an LH surge. The mutants had an increased rate of mid-gestation fetal resorption and pregnancy failure at full term, although this was previously reported to have disappeared from this colony (Low-Zeddies and Takahashi, 2001). One explanation given for the pregnancy loss was that lower estradiol levels observed in the mutants were responsible for the failure of *Clock* mutants to initiate labour. Interestingly estradiol administration failed to initiate an LH surge in ovariectomized *Clock* mutants despite a normal response to GnRH administration. In the former experiment, however, the steroid was administered and blood samples obtained at the same time of the day (at lights on) for both genotypes, which failed to take into account for the differences in residual rhythmicity reported in the two genotypes (Kennaway *et al.*, 2003a). Despite these results, the reproductive phenotype of the *Clock* mutant is at best only minimally compromised. How the mice can ovulate in the apparent absence of an LH surge is not known and probably unprecedented.

In our laboratory we have monitored the reproductive performance of a *Clock* mutant colony derived from the original Vitaterna colony, as well as a colony of melatonin-proficient (Kennaway *et al.*, 2003a) *Clock* mutants (Kennaway *et al.*, 2004). We found that both colonies could be efficiently maintained through homozygous pairing. Upon acute introduction of males, both *Clock* mutant lines took 2–3 days longer to mate and deliver pups compared to their wild type control lines. Litter sizes were slightly but significantly reduced in *Clock* mutant mice (seven versus eight pups). While similar proportions of wild type and mutant mice failed to produce live births within 40 days of pairing, survival to weaning was poorer in both mutant lines (80–84%) compared to 94–96% survival in wild type lines. Our experience therefore indicates that the *Clock* mutation has significant, but subtle, effects on reproductive performance.

In view of the prominent role of circadian rhythmicity in estrous cycle function and CLOCK protein in the generation of rhythms, the subtle effects of the *Clock* mutation are difficult to explain. By introducing functional copies of the genes for the melatonin synthesizing enzymes AA-NAT and HIOMT [known to be mutated in all mice strains other than CBA and C3H (Goto *et al.*, 1989)] into a *Clock* mutant background, we have shown that central, hypothalamic rhythmicity (i.e. the SCN signalling to the pineal gland to synthesise melatonin) is maintained in these mutants (Kennaway *et al.*, 2003a). We argued that this may occur through the recruitment of NPAS2 to partner BMAL1 and initiate the clock gene rhythms at least centrally, although peripheral rhythmicity is lost (DJ Kereneway and A Voultsios, unpublished results).

The other key clock gene, *Bmal1*, has been targeted and a null mutant line produced (Bunger *et al.*, 2000) and the evidence provided in that report suggested that it is a non-redundant partner of CLOCK despite the existence of a *Bmal2* gene. Although it was not stated explicitly in the original description of the *Bmal1* knockout line, it was stated subsequently that the homozygous null mutants are fertile (Cowden and Simon, 2002). Having established our own colony of *Bmal1* null mutants from the original Bradfield laboratory colony, we have found this declaration of fertility to be incorrect (Boden and Kennaway, 2004). We have never observed live births from homozygous pairings of *Bmal1* null mutants. Nevertheless the *Bmal1* females ovulate (following irregular cycles), mate and fertilize ova following mating with wild type males, but they have either delayed embryo development or early embryo loss, such that no full term pregnancies have ever been achieved in our colony.

Circadian rhythms and early pregnancy

Following fertilization, the embryo spends up to 4–5 days traversing the oviduct prior to entering the uterus. There is considerable evidence that the environment within the oviduct plays a significant protective and nutritive role in embryo development (Leese *et al.*, 2001). The importance of the environment during this period is evidenced by the enormous efforts to develop optimal supportive culture media for human *in vitro* embryo culture (Summers and Biggers, 2003). The role of circadian rhythmicity

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in early embryo development does not appear to have been widely studied. Delayed mating from 06:00 to 08:00 compared with 24:00 to 02:00 significantly decreased the percentage of morphologically normal embryos and increased the percentage of degenerated embryos (Sakai and Endo, 1988). While these deleterious effects may have been due to the delay in fertilization, they could also have been caused by the embryos reaching developmental milestones at inappropriate circadian times. Similarly it has been shown that the circadian period during early pregnancy influenced embryo development, since mice exposed to 22 or 26 h days had significantly higher percentages of dead or resorbed embryos 12 days post fertilization (Endo and Watanabe, 1989).

We have recently investigated rhythmicity in the rat oviduct using real-time RT–PCR analysis of clock genes and some selected functional genes (Kennaway *et al.*, 2003b). The oviduct is like most other peripheral tissues and expresses clock genes rhythmically. We also observed that plasminogen activator inhibitor 1 (*pai-1*) gene expression is rhythmic. The promoter for the *pai-1* gene is known to have the appropriate E-box for CLOCK/BMAL1 heterodimer binding (Maemura *et al.*, 2000; Schoenhard *et al.*, 2003) and so the rhythmicity is likely to be due to a direct effect of the clock gene rhythm in the oviduct cells. PAI-1 has been identified previously in the oviduct and is thought to play a role in protecting the embryo from protease damage during the traverse along the oviduct (Kouba *et al.*, 2000). If the circadian change in *pai-1* mRNA is reflected by changes in active protein, the embryo may experience periods of greater or lesser vulnerability to damage during development (Figure 3). Mis-matching of oviductal PAI-1 activity and stage of embryo development (for example, following delayed mating) may decrease viability. Other functional genes may be rhythmically expressed in the oviduct and influence embryo development.

Embryos express *Bmal1*, *per1* and *cry1* at all stages from oocyte to blastocyst (Johnson *et al.*, 2002). Expression of *Clock* was apparent up to the 2-cell stage, then decreased below the assay sensitivity and reappeared later at the blastocyst stage. There have been no studies on the role/influence of rhythmicity on implantation events.

Humans

Despite the very strong evidence for a critical role of the circadian timing system in determining the time of ovulation in animal models, there have been few studies conducted in humans. The human SCN has, however, been shown to contain estrogen receptor α and β and progesterone receptors (Kruijver and Swaab, 2002). The possible role of the SCN in ovulation in humans could be inferred from studies on the timing of the LH surge. In such a study in women who were being monitored prior to natural cycle IVF with 4 hourly blood sampling from day 9, the surge occurred between 24:00 and 04:00 in 37% of the cycles and between 04:00 and 08:00 in 48% of the cycles (Cahill *et al.*, 1998). In a subsequent report also using 4 hourly blood sampling in normal cycling women, 15 of 19 subjects 'initiated' their LH surge at 08:00 (Kerdelhue *et al.*, 2002). These authors attempted to link the timing of the surge to the time of the SCN-mediated cortisol peak and found that four of four women with a cortisol peak at 04:00 initiated the LH surge at that time. Of the 15 women initiating the LH surge at 08:00, 12 had their cortisol peak at the same time while the remaining three had peak cortisol at 04:00. This latter study provides the strongest evidence, albeit indirect, that SCN may be involved in the timing of ovulation in humans. More rigorous studies are needed to definitively prove this link and to establish whether rhythm disruption affects the timing of the LH surge and ovulation. As for the actual time of day that the oocyte is released, to our knowledge this has never been reported, presumably because the procedures are so intrusive.

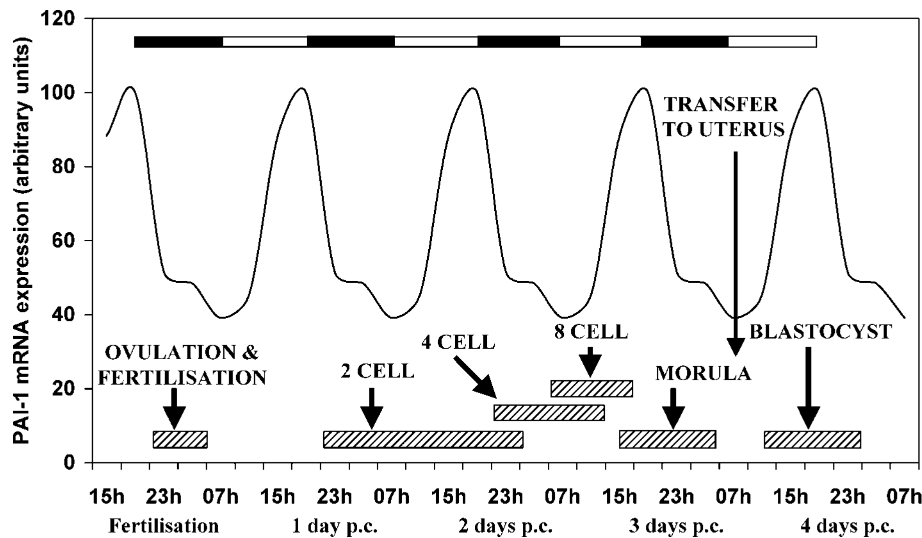

Figure 3. The relationship between the stages of mouse embryo development, the light dark cycle and oviduct expression of a putative embryo protective gene, *pai-1*. The light/dark cycle is illustrated by the closed and open bars at the top of the figure. The stages of embryo development are approximate boundaries of the various transitions. The graph shows the hypothetical *pai-1* mRNA rhythm in the oviduct during early pregnancy, based on a study in rat oviduct (Kennaway *et al.*, 2003b). PAI-1 is an example of many proteins expected to be rhythmically secreted into the oviduct that affect embryo development. It might be expected that perturbations in oviduct/uterus rhythmicity will alter embryo development. Similarly transfer of embryos to the oviduct/uterus at an inappropriate time of day for the stage of development may also compromise the embryo.

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In humans we might expect that some clues to the role of daily rhythmicity on early pregnancy could come from studies of women working shifts and/or travelling regularly across time zones. Shiftwork disrupts sleep rhythmicity and even those shiftworkers who do manage to establish daytime sleep patterns, for many the melatonin rhythmicity remains nocturnal (Benhaberou *et al.*, 1999). As a further example, a recent study reported an inverse relationship between the number of nights worked and melatonin levels (Schernhammer *et al.*, 2004). Since melatonin is widely recognized as an accurate marker of SCN function, this study provides compelling evidence that the SCN of these shiftworkers was affected by their working conditions. Although no human studies have been conducted as yet, animal experiments indicate that timing of food presentation affects liver and other tissue rhythmicity without affecting central rhythm timing (Damiola *et al.*, 2000). Thus shiftworkers who eat during the night when their SCN is maintaining normal melatonin secretion are likely to have conflicting central and peripheral cellular rhythmicity. The extent that the human reproductive axis is affected by shiftwork can really only be determined from epidemiological studies. Bisanti *et al.* (1996) attempted to analyse the effect of shiftwork on fecundity by recording the time of unprotected intercourse prior too pregnancy. They stated that despite some possible confounding factors, data from their study 'are in favour of an association between shiftwork and prolonged waiting time to pregnancy'. A later, larger similar study, however, concluded that there 'was no unequivocal evidence of causal association between shift work and subfecundity' (Zhu *et al.*, 2003). The small reduction they did observe, it was argued, may have been mediated by pregnancy planning bias or differences in opportunity for sexual contact. The evidence for a deleterious effect of shiftwork and time zone travel on pregnancy outcome is stronger (Cone *et al.*, 1998; Aspholm *et al.*, 1999; Knutsson, 2003) with an increased incidence of miscarriage, low birthweight and preterm birth. To what extent these adverse outcomes are the result of disrupted circadian timing rather than other lifestyle factors, including altered diet and stress, cannot be determined from these epidemiological studies.

Conclusion

The discovery of clock genes, the recognition of their widespread influence as transcription factors and their influence on a large number of functional genes should rekindle interest in the potential role of circadian rhythms in reproduction, including human reproduction. Clock genes are highly conserved from plants to *Drosophila* to rodents to humans. Given the role of rhythms in reproduction of other animals and the role of clock genes in the control of rhythmicity, it would seem unwise to ignore them in the quest for understanding and correcting fertility in humans. There is already emerging evidence that mutations in clock genes can alter human physiology and that polymorphisms can affect sleep behaviour. Contrary to previous beliefs, humans can respond to changes in photoperiod (Wehr *et al.*, 1993) and so it is no longer valid to presume that circadian rhythms are of little importance in human physiology, especially reproduction. Humans are notorious for poor fertility as evidenced by the huge demand for assisted reproductive technologies and yet we still do not know when the LH surge commences with a precision of $<12-24$ h, and we have little information on the actual time of day that ovulation occurs. Furthermore, assisted reproductive technologies involving *in vitro* culturing or manipulation of embryos and subsequent transfer to the uterus tend to ignore the fact that the processes are occurring in a non-rhythmic environment. Similarly in the case of transfer, the embryo is returned potentially out of phase with the mother as a result of slower development *in vitro*. It is certainly time to consider seriously the temporal aspects of reproduction.

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