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Entrainment of the circadian clock in humans: mechanism and implications for sleep disorders.

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Humans exhibit behaviour and physiology controlled by a circadian clock. The circadian period is genetically determined and administered by a series of interlocked autoregulatory feedback loops largely in the suprachiasmatic nuclei of the hypothalamus. The phase of the clock is, however, synchronised by a number of external environmental cues such as light. A failure or change in any one of the requisite clock components may result in the onset of a long-term sleep disorder. This review discusses the mechanism regulating circadian physiology in humans and explores how disturbances of this mechanism may result in sleep pathologies.

Key words: Circadian rhythm, sleep disorders, sleep phase syndrome, dyssomnia, jet lag syndrome

1. Introduction

Like other organisms, humans exhibit rhythmical behaviour indicative of an intrinsic biological clock. Humans experimentally deprived of environmental stimuli continue to exhibit sleep/wake cycles with ~24hr periodicity (Eysenck, 2004). Less obviously, physiological processes such as vasodilatation, heart rate and endocrine secretions are also under circadian influence (Hastings et al., 2003).

Zeitgebers are environmental cues that regulate the cycle of a biological clock. The 24hr periodicity of the human clock suggests that this system functions to facilitate anticipation of the solar day. Although the human clock may be entrained by sound, temperature, and social cues such as feeding, light is the most powerful zeitgeber known to synchronise the clock (Lowrey and Takahashi, 2000). Once light has been detected through the retina, intensity and duration are transduced by the hypothalamus into a biochemical mechanism based on autoregulatory feedback processes. Positive and negative components then regulate cellular processes through a series of molecular interactions to maintain 24hr rhythmicity.

2. The mechanism of entrainment

Entrainment of a biological clock is the process of determining both its period (i.e., 24hrs in most humans) and its phase. The latter refers to the offset of a circadian clock with respect to the standard 24hr cycle (Howe, 1993). In general terms, the period of the clock is genetically determined, whereas its phase is heavily influenced by environmental zeitgebers such as light.

2.1 Light is detected by a specialised mechanism through the retina

Light is primarily detected through the retina. This mechanism has been elucidated using mice homozygous for the *Retina degenerative* (*Rd*) mutation. After 70 days, these mice are judged by electron microscopy to have reduced cone and zero rod photoreceptors (Carter-Dawson et al., 1978). Indeed, *Rd* -/- mice are blind according to electroretinogram observations on exposure to bright light. Despite this visual blindness, photic stimuli have been found to influence the clock in *Rd* -/- mice (Lowrey and Takahashi, 2000). However, mice with eyes surgically removed were unable to detect light (Foster and Hankins, 2002). Taken together, these findings suggested the existence of a third

photoreceptor operating independently of rods and cones.

2.2 Melanopsin-expressing cells relay light signals to the hypothalamus

Novel photoreceptor cells were identified when retinal ganglion cells (RGCs) were shown to depolarise in response to light despite chemical retardation of synaptic input (Berson et al., 2002). Other researchers simultaneously characterised melanophores found in the photosensitive melanocytes of frog skin. The mammalian homologue of one such photopigment, melanopsin, was later shown using a βgal marker gene to be expressed solely in RGCs. Murine knockouts of the melanopsin gene, however only partially influenced phase delays of the circadian clock (Ruby et al., 2002). Strains of *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ mice were then used which are deficient in melanopsin but also degenerate for rod-cone phototransduction mechanisms. These triple knockout mice could not entrain to light-dark cycles (Hattar et al., 2003). This implies that the melanopsin and rod-cone pathways are complementary with regards to entraining the biological clock.

2.3 RGCs transduce light into an electrical signal detected by the hypothalamus

Intravitreal injection with a strain of pseudorabies virus showed that this marker was transported transsynaptically to the suprachiasmatic nuclei in the hypothalamus (Smeraski et al., 2004). This structure is known to be associated with the biological clock as pituitary tumours that compress this region destroy clock function in humans. Ablation of suprachiasmatic nuclei in mice similarly abolishes murine expression of circadian rhythms. Temporal behaviour in these mice is, however, restored by grafting neonatal hypothalamic tissue onto the ablated region (Hastings, 1998). This evidence strongly implicates suprachiasmatic nuclei involvement in the mammalian biological clock. The location of this structure is illustrated by Figure 1.

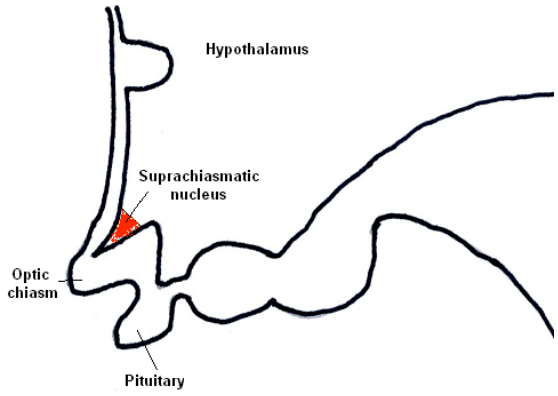

Figure 1. The 10,000 neurones of the SCN are located around the midline of the optic chiasma in the temporal lobe. Image modified from Bear et al., 2001.

2.4 Neuron excitation stimulates a mechanism of negative feedback oscillations

Neurons of the suprachiasmatic nuclei depolarise independently, as shown by the rhythmic electrophysiology of rat neurons cultured in isolation (Welsh et al., 1995). This cell-autonomous nature of rhythm in the suprachiasmatic nuclei implicates a genetic component. Figure 2 shows how the human biological clock might function at the molecular level. Indeed, the firing of SCN neurons is thought to activate genes through a Ca^{2+}-dependent kinase cascade. This induces expression of clock genes such as *Per1* and *Per2,* which carry Ca^{2+}/cAMP response elements (Hastings et al., 2003).

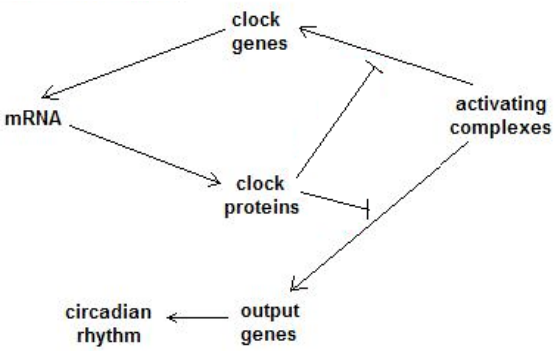

Figure 2. Biological clocks are typically based on negative feedback mechanisms that cause oscillations in output gene expression. In *Drosophila*, negative regulators occur in the form of a complex composed of the proteins Period and Timeless.

2.5 The CLOCK-BMAL1 complex is a positive clock regulator

Chemical mutagenesis in mice revealed a gene which, if disrupted, extended the circadian period (Vitaterna et al., 1994). Mice homozygous for this mutation further became arrhythmic in constant darkness (Shearman et al., 2000). This *Clock* (*Clk*) gene encodes a bHLH/PAS family transcription factor that has been identified by transgenic bacterial artificial chromosome (BAC) rescue (Antoch et al., 1997). Indeed, the *Clk* mutation results in a 51-residue deletion from the transcriptional activation domain (Shearman et al., 2000). The CLOCK protein has been shown by use of a yeast-2-hybrid system to dimerise with another bHLH/PAS transcription factor, BMAL1. The protein BMAL1 is also an essential clock component as *Bmal1* knock out mice are arrhythmic (Bunger et al., 2000). Murine *Bmal1* is rhythmically expressed in the SCN, peaking at ~15 hours after dawn (CT15) whereas rat *Clk* expression can be light induced (Lowrey and Takahashi, 2000). The CLOCK-BMAL1 complex mediates transcription through CACGTG E-box enhancer elements. The use of a dominant negative CLOCK protein has shown that targets of the CLOCK-BMAL1 complex include the *Per* and *Cry* genes (Gekakis et al., 1998). Although able to heterodimerise with BMAL1, the mutant CLOCK protein is unable to regulate transcription and consequently resulted in *mPer1*, *mPer2*, *mPer3* and *mCry1* transcript deficiencies (Shearman et al., 2000).

2.6 Per and Cry genes encode negative clock regulators

Three mammalian *Period* (*Per*) genes were cloned by exploiting PAS sequence homology with *Drosophila* (Tei et al., 1997). The *mPer1* mRNA abundance determined by quantitative *in situ* hybridisation revealed a peak at midday in the suprachiasmatic nucleus. The light-induction of *mPer* was confirmed by light pulses in the dark that were followed by increased abundance of *mPer* transcripts (Shigeyoshi et al., 1997). Series confocal microscopy has since localised immunoreactive mPER to the cell nucleus, suggesting a role for mPER in regulating transcription (Hastings et al., 1999). Indeed, detection of immunoreactive nuclear mPER-ir is phase lagged behind expression of *mPer* mRNA (Maywood et al., 1999). These lines of evidence are consistent with the negative feedback loop model and suggest a role for mPERs as regulators of the human clock. Disruption of each *mPer* gene in mice has refined this functional definition. Indeed, *mPer1*/*mPer3* and *mPer2*/*mPer3* double mutants exhibited rhythms similar to *mPer1* and *mPer2* mutants respectively. This showed that *mPER3* functions outside of the primary clock mechanism. Indeed, *mPer1*/*mPer2* double mutants were arrhythmic (Bae et al., 2001).

Cryptochrome genes such as *mCry1* and *mCry2* encode products related to photolyase DNA repair enzymes (Hsu et al., 1996). These flavoproteins are particularly highly expressed within the ganglion cell layer and inner retina. Although not directly influenced by light pulses, expression of both *mCry1* and *mCry2* exhibit circadian rhythmicity (Lowrey and Takahashi, 2000). It is known that *mCry* genes are essential components of the clock as *mCry1* -/- *mCry2* -/- mice exhibit arrhythmic wheel running in darkness (Shearman et al., 2000). Indeed, the roles of mCRYs and mPERs in the mammalian clock are to promote mutual stabilisation and nuclear transfer (Hastings and Maywood, 2000). Once in the nucleus, both mCRYs and mPERs independently repress CLOCK-BMAL1-mediated transcription, as shown by *in vitro* luciferase reporter gene assays (Shearman et al., 2000). Furthermore, in *mCry1* -/- *mCry2* -/- mice, both *mPer1* and *mPer2* are expressed arrhythmically and at high levels in the SCN (Okamura et al., 1999). This is consistent with the luciferase observations as mice lacking mCRYs would show reduced repression of CLOCK-BMAL1-mediated mPer transcription (Okamura et al., 1999). As CLOCK-BMAL1, mPERs and mCRYs are inter-regulated, this suggests a mechanism whereby *mCry* and *mPer* may autoregulate their own expression.

2.7 The result of Per/Cry and Clock-Bmal1 interactions is a molecular oscillator

The result of these interactions is a pair of negative feedback loops that communicate with one another (Yu et al., 2002). This

communication occurs through the orphan nuclear receptor REV-ERBα which is negatively regulated by CRYs/PERs and positively regulated by the CLOCK-BMAL1 complex. In turn, REV-ERBalpha represses *Bmal1* transcription (Shearman et al., 2000). Support for this model comes from *mCry1* and *mCry2* knockout mice, which suffer enhanced but arrhythmic *mPer* expression (van der Horst et al., 1999). As shown in Figure 3, the result of these interlocking feedback loops is a series of molecular oscillations.

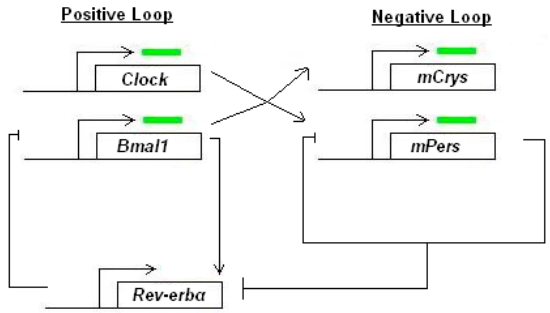

Figure 3. Two feedback loops communicate with one another through Rev-erbalpha. The result is molecular oscillations that set the phase of the clock.

2.8 24hr periodicity is determined by phosphorylation of PER and CRY

The *Tau* mutation was first identified in the Syrian hamster as a semidominant mutation that shortens the clock period by 2hrs in heterozygotes and by 4hrs in homozygotes (Gekakis et al., 1998). Using positional cloning and syntenic information from the human genome, researchers tracked this mutation to a gene encoding casein kinase ε(CKIε). This was confirmed by disrupting this gene in hamsters, which results in the *Tau* phenotype (Lowrey and Takahashi, 2000). Murine PER (mPER) was later shown to be a phosphorylation target for CKIε (Akashi et al., 2002). This was established by immunoprecipitation which demonstrated interaction between CKIε and mPER1 (Lowrey and Takahashi, 2000). Using molecular mass as a marker for phosphorylation, it was shown that *Tau* mutant CKIε markedly reduced phosphorylation of mPER *in vitro* (Ralph and Menaker, 1988).

2.9 Phosphorylation retards nuclear localisation of mPER1

As well as destabilising mPER1, phosphorylation by CKIε also affects its localisation. Isolated expression of mPER1 in HEK293 cells demonstrated that this protein is localised to the nucleus. Nuclear translocation of mPER1 is favoured by mPER2, which co-localises following heterodimerisation with mPER1. Nuclear localisation is, however, delayed by CKIε that, when co-expressed with mPER1, phosphorylated mPER1 and masked its nuclear localisation signal (Vielhaber et al., 2000). Phosphorylation was implicated in this process because CKIε exhibiting an inactive kinase domain failed to retain mPER1 in the cytoplasm. Indeed, a related kinase isoform, CKIΔ, can also phosphorylate mPER1 and retard its nuclear localisation (Lowrey and Takahashi, 2000). Interestingly mPER3 only translocates to the nucleus in a CKIε-dependent manner. When potential mPER3 phosphorylation sites were mutated, nuclear translocation was markedly reduced (Akashi et al., 2002).

2.10 Phosphorylation destabilises hPER1

Phosphorylation has also been shown to destabilise human PER1 (hPER1). Indeed, unphosphorylated hPER1 has a half life of ~24hrs, which is over twice that of phosphorylated hPER1 (Ralph and Menaker, 1988). This is consistent with the hamster *Tau* phenotype, which exhibits a markedly reduced circadian period.

Given the ubiquitin-mediated proteasomal degradation of TIM in *Drosophila*, it has been suggested that phosphorylation of hPER1 might exert a similar effect (Lowrey and Takahashi, 2000). Ubiquitination is a three stage process in which molecules of the 76-residue polypeptide ubiquitin are added to a lysine residue of the target protein. This marker is recognised by proteasomes which proteolytically cleave the marked protein in an ATP-dependent manner (Lodish et al., 2003). The application of this process to the mammalian clock is supported by disruption of potential phosphorylation sites on mPER3, which reduced proteasomal degradation (Akashi et al., 2002).

2.11 The result of these feedback loops is a clock with 24hr periodicity

The human circadian clock is based on a number of interacting mechanisms that regulate transcription, translation and post-translational modifications (Hastings and Maywood, 2000).

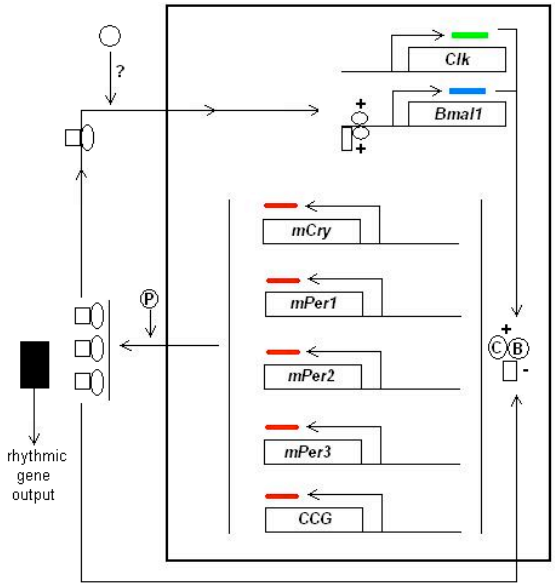

Figure 4. As *Clk* and *Bmal1* are transcribed, their protein products heterodimerise. The resulting CLOCK:BMAL1 complex then up-regulates transcription of the clock-controlled genes (CCGs): *Crys* and *Pers*. CLOCK:BMAL1 mediated transcription is, however, repressed at night by CRYs and PERs. When transcription does occur, however, the gene products can result in a rhythmic output. This may further be regulated by CKIε which destabilises and represses PER1 by phosphorylation. Image modified from Shearman et al., 2000.

Based in the SCN, the mechanism of this central clock is summarised in Figure 4. The result of this program in molecular terms is shown in Table 1.

Table 1: Circadian rhythm associated with each clock component (Lowrey and Takahashi, 2000)

Component	Circadian rhythm
Per1	Transcription begins just prior to dawn, peaks 6hrs later then shows rapid decline. Protein follows the same rhythm but 6hrs after transcription.
Per3	Transcription high in the morning until ~6hrs after dawn. Declines after dusk.
Per2	Transcription occurs later than Per1 and Per2. Peaks at dusk.
Cry1/*Cry2*	Peaks at ~8hrs after dawn then declines.

When translated, PER1 translocates to the nucleus and represses the CLOCK-BMAL1 complex from up-regulating *Per1* transcription (Vielhaber et al., 2000). The consequence of this repression is that CLOCK-BMAL1 is inactivated at night resulting in decreased transcription of *Per* and *Cry* (Lowrey and Takahashi, 2000). De-repression occurs in the form of CKIε-mediated PER1 phosphorylation which prevents nuclear localisation and marks PER1 for proteasomal degradation (Ralph and Menaker, 1988). In this way, CKIε regulates the autoregulatory loop by controlling protein turnover and nuclear localisation (Akashi et al., 2002). This control resets the process and allows the cycle to begin again at dawn (Lowrey and Takahashi, 2000). As shown by Figure 5, the application of this program to physical time has been neatly demonstrated using mPer::Luciferase transgenic mice and bioluminescent imaging. These techniques provided a realtime image of the murine clock ticking (Yamaguchi et al., 2001).

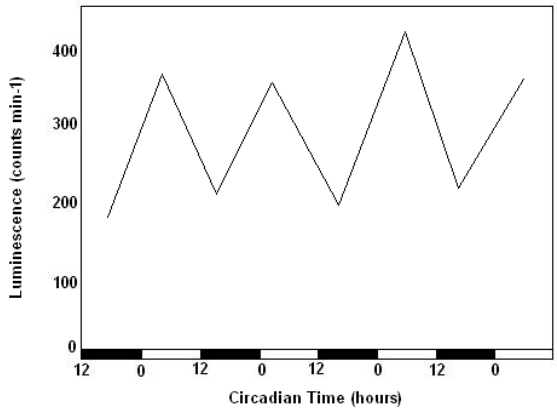

Figure 5. An approximation of bioluminescence imaging used to follow the rhythmic expression of the *Per1* gene in the mouse. Image modified from Yamaguchi et al., 2001.

2.12 Cells peripheral to the SCN may exhibit their own circadian rhythms

cDNA microarray technology has been used to demonstrate circadian transcriptome rhymicity in murine peripheral tissues (Akhtar et al., 2002). Indeed, 9% of sampled liver transcripts and 5% of those sampled from the heart were circadian (Lowrey and Takahashi, 2000). Similarly cultured rat fibroblasts treated with high concentrations of serum induced circadian gene expression. These genes encoded Rev-Erbalpha among other components associated with the mammalian clock. In these experiments, serum shock appeared to mimic the effect of light pulses on gene expression in the SCN (Balsalobre et al., 1998).

2.13 Peripheral circadian clocks have limited autonomy from the SCN

Transgenic rats whose tissues express luciferase *in vitro* have been used to study the relationship between SCN and peripheral clocks. Restricting food availability did not affect SCN phase but altered the liver rhythm by 10 hours in 2 days (Stokkan et al., 2001). This demonstrated some degree of autonomy of the peripheral tissues from the SCN circadian clock in response to environmental cues. Microarray analysis has shown however that peripheral rhythms are severely disrupted on surgical ablation of the SCN from mice (Akhtar et al., 2002).

A hierarchical model has been proposed to accommodate these observations. Synchronised to solar day, the primary SCN is considered to be the primary neural oscillator controlling the mammalian clock. The SCN is linked, however, to local clocks in peripheral tissues by both neural and endocrine factors (Hastings et al., 2003). This confers flexibility on the system so that local environmental cues (e.g., food) can be accommodated without interfering with the primary circadian clockwork.

3. Implications for jet lag and sleep disorders

The preceding description of clock entrainment may be reduced to three components. These are an input pathway based on environmental cues, a genetically determined central oscillator, and an output pathway of physiological behaviour (Koyanagi and Ohdo, 2002). Factors disrupting any of these components may shift the clock phase or circadian period and result in an abnormal sleep/wake cycle.

3.1 Sleep phase syndromes are primarily genetic sleep disorders

Sufferers of the hereditary familial advanced sleep-phase syndrome (FASPS) possess an accelerated clock. Indeed, their circadian period is ~20hrs as opposed to the normal ~24hr cycle (Reid et al., 2001). Using linkage analysis, researchers associated a single mis-sense point mutation in the human *Per2* gene with onset of FASPS. In the mutated hPER2 protein, the serine at position 662 in the CKIε-binding site is exchanged for an inactivating glycine (Toh et al., 2001). This results in hypophosphorylation of hPER2 and consequently reduced proteasomal degradation in the SCN. The resulting accumulation of hPER2 reduces the circadian period by disrupting the feedback mechanism. Researchers later identified another mutation that can result in FASPS. A threonine to alanine mis-sense mutation in CKIΔ was shown to reduce the circadian period in mice which is a phenotype reminiscent of FASPS (Xu et al., 2005). Interestingly, this is similar to the *Tau* mutation in hamsters, which

shortens the circadian period by disrupting CKIε (Gekakis et al., 1998).

Delayed sleep phase syndrome (DSPS) has the opposite effect and so lengthens the circadian period. Around 50% of relatives of sufferers exhibit symptoms associated with DSPS (Maret and Tafti, 2005). An unusually short allele of the human *Per3* gene has been associated with DSPS using the polymerase chain reaction (Archer et al., 2003). The molecular consequences of this length polymorphism at the *Per3* locus have not yet been established.

3.2 Narcolepsy is brought about by both genetic and environmental factors

Narcolepsy is a neurological disorder the major symptoms of which include daytime tiredness and cataplexy (Kok et al., 2002). Monozygotic twin studies show a concordance for narcolepsy of ~30%, which indicates a genetic predisposition but also an environmental cause (Maret and Tafti, 2005). Indeed, narcolepsy has been associated with deficiency of the hormone hypocretin as shown by canine mutations in the hypocretin receptor 2 gene which result in a narcolepsy-like phenotype (Kok et al., 2002). Hypocretin secretion from the posterior hypothalamus is controlled by the SCN and so is encoded by a clock output gene. Indeed, rats with surgically ablated SCN show uncharacteristically arrhythmic hypocretin-1 section (Zhang et al., 2004). Hypocretin-1 in turn regulates other hormones that affect alertness such as histamine, adrenaline, acetylcholine, dopamine, monoamine oxidase and leptin (Kok et al., 2002). In narcolepsy, then, the breakdown appears to occur immediately outside of the biological clock. Indeed, the association of narcolepsy with leukocyte antigen subtype DQB1*0602 suggests the disorder is caused by autoimmune destruction of hypocretin-secreting neurons (Kok et al., 2002). In this case, signals from the clock would be denied a target through which to increase hypocretin secretion.

3.3 Jet lag is a result of changes in the external light/dark cycle

Although clock periodicity in healthy humans is set to ~24hrs, phase is determined by light detection through the retina. When humans travel long distances to different timezones, the biological clock does not immediately reset and is said to be out of phase. This can be disorientating as the new environment may demand alertness at times when the biological clock is preparing for sleep. Disruption of the sleep/wake cycle in this way may result in insomnia and fatigue. Eventually the new light/dark cycle will reset the clock and entrain its phase to the new environment. A similar pattern of insomnia and fatigue may be observed in shift workers and for similar reasons. In the case of shift workers, the biological clock is entrained primarily by behaviour and takes around a week to adapt to a new shift pattern.

Like hypocretin in narcolepsy, melatonin (N-acetyl-5 methoxy-tryptamine) exhibits circadian rhythmicity. Melatonin secretion is indirectly controlled by the SCN through activation of N-acetyl-transferase in the subjective night time (Fukuhara et al, 2005). Indeed, orally administered melatonin taken prior to bedtime in the new timezone has been shown to reduce jet lag when compared to a placebo (Herxheimer and Petrie, 2001). Melatonin has also shown some success in entraining the circadian clock of those blind people who lack all light perception (Lewy et al., 2004).

4. Conclusion

Like all biological clocks, the human clock passes input stimuli through a central oscillator before producing output physiology or circadian behaviour. Entrained in part by zeitgebers such as light, periodicity and phase are maintained through a series of interlocking autoregulatory feedback loops in the SCN. These in turn delay the clock by affecting the transcription, translation and post-translational modification of additional clock components.

Aside from this central clock, cells in peripheral tissues may maintain their own circadian rhythmicity. Although dominated by the SCN, these peripheral clocks may be locally entrained to non-photic stimuli such as feeding.

With so many interactions between environment, neurons, genes and gene products, the clock mechanism is sensitive to mutation and large-scale environmental changes. When the clock cannot accommodate such changes, possible consequences include jet lag and sleep disorders. These pathologies highlight the fine balance between success and failure maintained by complex physiological systems. In reverence of this balance, the SCN has been dubbed by the popular press 'the brain's Timex' (Shipley, 2003). In light of its robust yet infinitively elegant complexity, this horological masterpiece is surely better described as 'the brain's Rolex'.

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