

The Circadian Clock and its Genes: in the Brain and Beyond

The holy grail of molecular neuroscience is to explain how the activity of specific suites of genes within defined neuronal populations leads to adaptive behaviour. Recent analyses of the molecular genetic and neural bases of circadian timing provide a cardinal example of such genes-to-cells-to-behaviour reductionism. In doing so, the lid has been lifted on a neuroscientific 'black box' to reveal a cellular timing mechanism that has a pervasive impact on most tissues of the body and, hence, on mental and physical well-being.

Circadian rhythms are those daily cycles of physiology and behaviour driven by an internal 'body' clock. They therefore continue to 'free-run' with a period of approximately (circa-) one day (dies) when an individual is experimentally isolated without time cues. Our most obvious circadian rhythm is the cycle of sleep and wakefulness, but this is complemented by a multitude of physiological rhythms many of which (epithelial cell division, hepatic detoxification and systolic blood pressure) have direct clinical relevance. In nature, these internal programmes are synchronised to each other and to the 24 hour solar cycle, enabling our physiology to adapt to the challenges and opportunities presented alternately by day and by night. In modern life, however, changing socio-economic demands, increased longevity and associated neurodegenerative disease disrupt this fine-tuning.¹ For example, shift workers experience increased incidence of metabolic diseases,^{2,3} whilst sleep disturbance is an extensive problem for the elderly⁴ and the principal cause of institutionalisation in Alzheimer's disease and related disorders⁵ (Figure 1).

The suprachiasmatic clock

The circadian pacemaker of the brain is the suprachiasmatic nuclei of the hypothalamus (SCN) (Figure 2a). When isolated in vitro these bilateral clusters of ca. 10,000 neurons continue to exhibit circadian cycles of metabolism, electrical firing, neuropeptide secretion and gene expression.⁶ Circadian time is not, however, an emergent property of a neural network. Many if not all SCN cells are autonomous clocks: a remarkable localisation of neural function. The ventral core of the SCN, characterised by GABA-ergic cells that co-express vasoactive intestinal polypeptide (VIP), receives afferents principally from the retina and brain stem that synchronise the clock to light and to social cues, respectively. The retino-hypothalamic pathway is especially intriguing because it contains projections from intrinsically photoreceptive retinal ganglion cells, which employ a novel photopigment melanopsin, and depolarise in response to light.⁷ Although retinal rods and cones can contribute to circadian entrainment, they are not necessary for it. The dorsal shell of the SCN, typified by GABAergic neurons that co-express arginine vasopressin (AVP), is synchronised by the core neurons: loss of inter-neuronal signalling via the VPAC2 receptor for VIP (Figure 2b) desynchronises the population of clock cells.⁸ Together, VIP and AVP-ergic projections from the SCN convey circadian signals, both directly and indirectly, to hypothalamic centres, the brain stem and spinal cord to control neuroendocrine and autonomic rhythms. Of especial interest in the context of sleep regulation are connections to the sleep centres of the ventral preoptic area and the orexin/ hypocretin neurons of the medial hypothalamus.⁹



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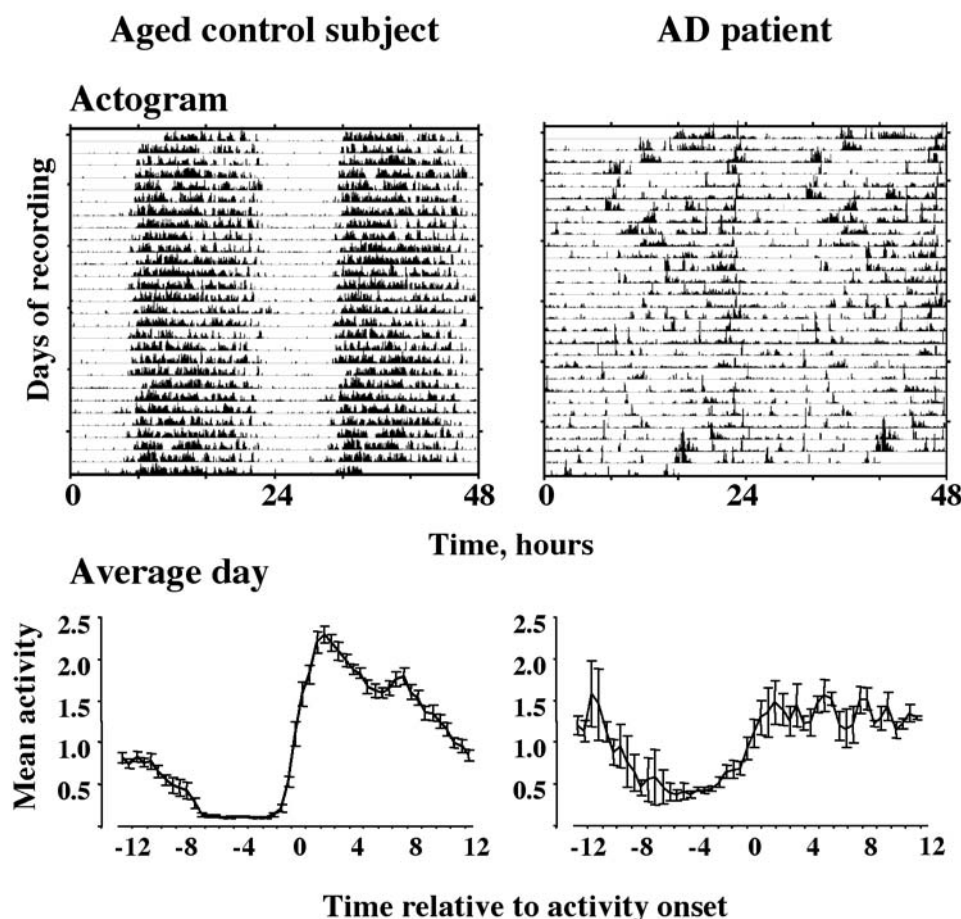


Figure 1: Disturbed rest/activity cycles in dementia
Rest/ activity records, obtained in home settings using wrist-worn activity meters, of representative healthy aged control subject and a patient with putative Alzheimer's disease (AD) with moderate dementia. Upper panels are daily records (actograms) plotted in 48 hour format for ease of inspection. Lower panels are graphical plots of the average daily cycle over the 4 weeks of recording. Note clear and robust 24 hour rhythm in age-matched control and loss of definition in patient, with nocturnal activity and loss of consolidated activity in daytime. Taken from reference 14.

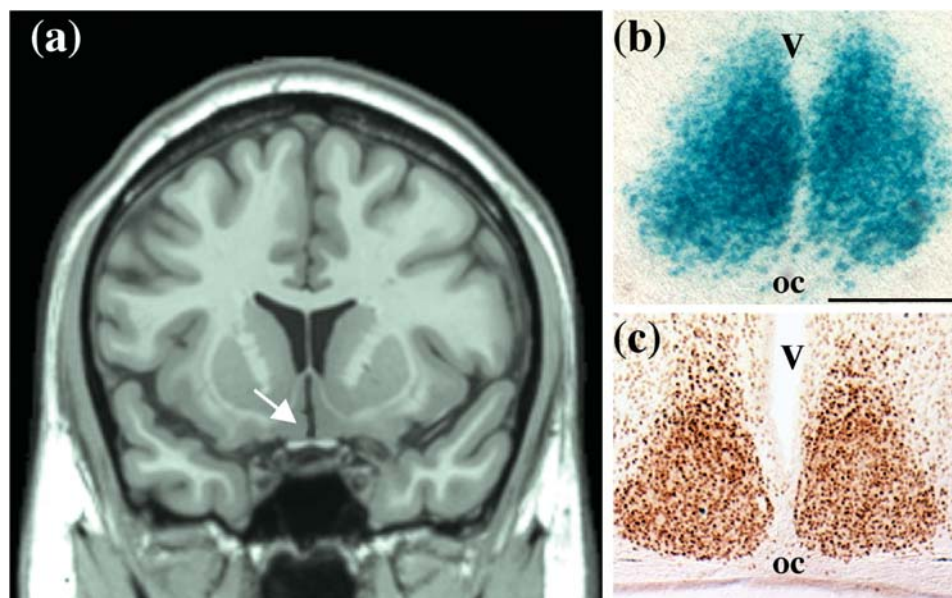


Figure 2: The suprachiasmatic nuclei as the brain's clockwork
 (a) Frontal MRI scan of human to illustrate position of SCN (arrowed) at conjunction of optic chiasm and third ventricle. Image courtesy of Dr Adrian Owen, MRC CBSU, Cambridge.
 (b) Frontal section of SCN from mouse carrying beta-galactosidase transgene as a reporter of the VPAC2 receptor for VIP. This neuropeptide receptor is widely expressed in SCN and is essential for circadian synchronisation of the clock cells. oc: optic chiasm, v: third ventricle, scale bar 500μm.
 (c) Frontal section of SCN from mouse immunostained for PER2 protein, a critical component of the molecular clockwork. At this stage of the circadian cycle, subjective dusk, almost all SCN neurons are expressing the PER2 protein in the nucleus where it negatively regulates *Per2* and other circadian genes.

Clock genes

Clues to the molecular genetic machinery of the SCN clock came from mutagenesis screens in the fruit fly, which identified first *period* and then *timeless* as 'clock genes'. Following the identification of mammalian homologues of the fly genes and the *de novo* discovery of the *Clock* gene by mutagenesis in mice, expression and biochemical studies have established a model of mammalian clockwork based upon an auto-regulatory, negative feedback loop involving both transcriptional and post-translational events^{9,11} (Figure 3a). In mammals, CRYPTOCHROMES (CRY) are the protein partners to PERIOD (PER). Expression of the two *Cry* and three *Per* genes is activated at the start of circadian day by transcriptional complexes containing CLOCK and BMAL proteins that act via E-box regulatory sequences on the *Per* and *Cry* genes. Towards the end of circadian day PER and CRY protein complexes accumulate in the nuclei of SCN cells (Figure 2c) and initiate the negative feedback phase, shutting off CLOCK and BMAL activity. Consequently, as PER and CRY are degraded and not replaced, their abundance decreases across circadian night, so that with the next circadian dawn the genes are released from negative feedback and the cycle able to restart. The stability, precision and amplitude of this cycle are enhanced by a secondary loop that involves two further rhythmically expressed proteins; RORA and REV-ERB α , which respectively activate and repress the *Bmal* gene. Their co-ordinated actions lead to rhythmic *Bmal* expression that peaks as *Per* and *Cry* expression reaches its minimum, thereby facilitating the initiation of the new cycle.

The molecular cycle is entrained by glutamatergic retinal afferents, which act via NMDA and AMPA receptors on SCN neurons to activate calcium dependent kinases, ultimately

leading to induction of *Per1* and *Per2* genes. Under steady state conditions, marginal induction of *Per* by dawn or dusk light will trigger a small phase shift that entrains the clock to exactly 24 hours and so matches it to external time. Travel between time-zones demands shifts of up to 12 hours but the molecular apparatus is unable to accomplish this in one cycle. Hence 'jet-lag' arises as a mis-match between internal physiological time and the external world. Realignment of internal time proceeds at the rate of approximately 1 h per day, although molecular delays are more rapid than advances, hence the relative ease of westwards travel. The debilitating effects of rotating shiftwork also have a circadian basis. Not only is sleep sub-optimal because it is scheduled to occur when the internal clock is promoting the waking state (and vice versa), but also the internal physiology of a worker can be in a permanent jet-lag like state over the course of the shift rotation.

Circadian clock mutants in mice and people

Because of the complex interactions within these feedback loops, mutations of the individual genes in mice are associated with various phenotypes.¹¹ An induced mutation of *Clock* compromises the trans-activating function of the encoded protein and lengthens circadian period from 23.5h in wild-type to 27–28h in the homozygote. Targetted knock-out of *Bmal* makes mice behaviourally arrhythmic, as does loss of *Per2*. In contrast, loss of *Per1* destabilises circadian behaviour but loss of *Per3* has little effect on rest/activity cycles. Loss of both *Cry* genes also leads to arrhythmicity, probably because in the absence of CRY proteins, PER2 protein is destabilised. Indeed, the stability of PER proteins appears to be a critical feature of the feedback loop, and phosphorylation plays a pivotal role in

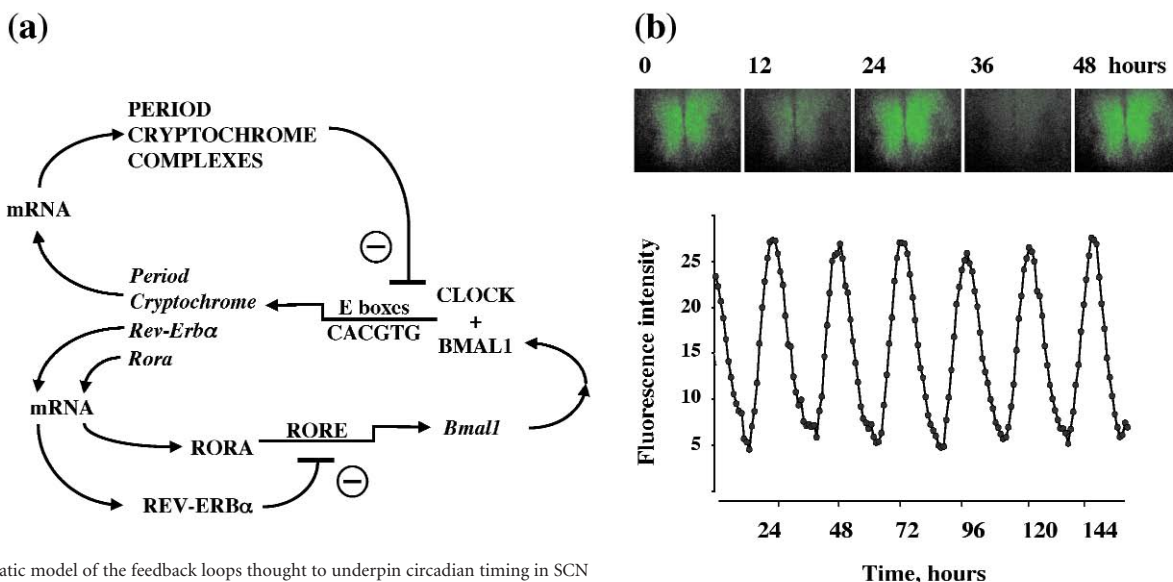
this. Studies in *Drosophila* have revealed that several kinases, including two casein kinases, phosphorylate *Per* and target it for proteosomal degradation, and that mutations to the kinases and associated ubiquitin ligase complexes alter circadian period and/or stability. In the Syrian hamster, a spontaneous mutation in the gene encoding casein kinase 1 ϵ dramatically accelerates circadian period to 20h in the homozygote. Changes in PER stability also have marked consequences in some people. Familial advanced sleep phase syndrome (FASPS) is a rare condition characterised by severely advanced sleep/wake cycles and circadian period of ca. 21–22 hours. It arises from mutations of critical phosphorylation sites in the casein kinase-binding domain of human PER2 protein,¹¹ or a mis-sense mutation in the kinase itself.¹² More generally, length polymorphisms in the *Per3* gene have been associated with evening and morning preference in the wider population.¹³

Real-time imaging of circadian timing in SCN

A major new technical development that has revolutionised our appreciation of the intrinsically dynamic nature of circadian mechanisms has been the advent of real-time imaging of circadian gene expression. Using organotypic SCN slices from rodents carrying fluorescent or bioluminescent reporter transgenes driven by circadian promoter sequences, it is now possible to observe the molecular clockwork 'ticking' through its cycle (Figure 3b). The most pronounced molecular cycles are concentrated in the AVP-rich region of the SCN shell. Under normal conditions the cell population is tightly synchronised, *Per* expression peaking in the circadian day with the medial neurons phase-leading by two to three hours. The remarkable precision and autonomy of this molecular timekeeper is emphasised by its persistence in organotypic SCN slices maintained in culture for many months: this is no feeble clock! Intercellular communication is, however, critical for normal function. Treatment with TTX to block Na⁺-dependent action potentials immediately suppresses the amplitude of circadian gene expression, and over several days the population of rhythmic cells becomes desynchronised. A comparable loss of amplitude and desynchrony is seen in mice lacking the VPAC2 receptor for VIP, emphasising the role of SCN neuropeptidic signals in sustaining intra-cellular timing.⁸

Circadian timing in peripheral tissues

As noted above, most physiological processes exhibit circadian modulation and in particular pathologies, most notably cardiovascular disease; this translates into a circadian prevalence of morbidity and mortality.¹ Such circadian co-ordination lies in temporal programming of gene expression, as revealed by micro-array based transcriptomic analyses, and also circadian co-ordination of the proteome. Until recently, the prevailing view was that exclusively SCN-dependent signalling drove these peripheral cycles. Real-time recording of circadian gene expression has now shown that many peripheral tissues, including heart, liver and kidney contain their own local clockwork, based on the same molecular feedback



(a) Schematic model of the feedback loops thought to underpin circadian timing in SCN neurons and other rhythmic cell types. Period and Cryptochrome are the principal genes mediating negative feedback. The cycle is stabilised by a secondary loop involving two orphan nuclear receptors, RORA and REV-ERB α which, respectively, activate and suppress expression of Bmal thereby timing the onset of activation to Per and Cry by CLOCK/BMAL complexes. The activity of clock-output genes will be timed by the rhythmic abundance and accumulation of PER, CRY, RORA and REV-ERB α and their dependent factors. See text for further details.

loops as in the SCN. The SCN are now viewed as a co-ordinator of these local clockworks rather than a primary driver of peripheral rhythms. Internal synchronisation is dependent on multiple redundant pathways, including corticosteroid secretion and metabolic cues related to feeding schedules. The SCN hold a privileged position because they are the sole entry point of photic information into the system, and because they directly or indirectly control the internal synchronising cues. Experimentally, for example with restricted feeding schedules, the link between SCN time and peripheral clocks can be broken. The prevalence of cardiovascular and gastrointestinal disease in long term-shift workers may be related to metabolic dysfunctions caused by inappropriate meal-times. Granny's dictum that regular sleep and three regular daily meals is the key to a long and happy life may well have circadian truth behind it.

Circadian disorder in neurological disease

The incidence of sleep disorders, many of unspecified origin, increases with age, especially above 60 years. Many will not have a circadian origin, although in a few definitive cases such as

FASPS, mutations of the molecular clockwork are clearly causal in disrupting the sleep pattern. The circadian timing of sleep in the healthy elderly is robust, even if the duration or quality of sleep is disappointing. Equally, in aged rats the molecular oscillator of the SCN, as reported by Per-dependent luciferase emission, is perfectly competent when isolated in culture. In neurological conditions, especially Alzheimer's disease (AD) and Huntington's disease (HD), the situation may be very different. The progressive loss of daily organisation to the sleep-wake cycle in AD and the problems this causes for patient care in a home setting is the principal cause of institutionalisation, with its incumbent personal, social and economic costs (Figure 1).¹⁴ In AD, it remains unclear as to whether the central timekeeper of the SCN is compromised. Some dependent rhythms such as core body temperature show alterations of phase and/ or amplitude, but are essentially rhythmic, as is cortisol secretion. The sleep disorder may therefore reflect a disturbance in signalling between the SCN clock and centres controlling sleep and arousal, or an inability within the cerebral cortex to sustain a sleeping and/ or waking state. Nevertheless, effective management of the sleep disruption, for example by

enhanced environmental cues (nocturnal darkness, bright daytime light, regular meals), could benefit patients and carers.¹⁵ Moreover, nothing is known of the peripheral clock functions in AD patients: are they running normally or are they and their dependent metabolic cycles compromised, leading to a general malaise?

Sleep disorder is also recognised in HD. Again this may be multi-factorial, but direct evidence for a circadian basis comes from transgenic mouse models, which recapitulate the disorder of increased activity during the normal sleep phase, blurring the behavioural distinction between day and night.¹⁶ Moreover, circadian gene expression in the SCN of these mice is normal when they are pre-symptomatic but becomes severely blunted as the rest/activity pattern breaks down. It remains to be determined whether disturbance of the molecular time-keeper arises from altered sleep patterns, or actually causes the behavioural abnormality. As with AD, neurodegeneration may compromise the transmission of circadian cues to sleep centres and/ or the forebrain. The general point nevertheless remains that effective management of 24 hour behaviour would benefit both patient and carer, even if the progression of the disease can not be curtailed.

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