THE CIRCADIAN CLOCK: ORCHESTRATING GENE EXPRESSION AND PHYSIOLOGY

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Timing is everything. How true is this proverb? Time has neither a beginning nor an end, and hence, it is difficult to define. What we experience as time is related to a reference point and, hence, relative. Living on earth has made us use the sun as reference and the 24-hour succession of light and darkness is probably the most pervasive epigenetic influence in the evolution from a single cell organism to man. This periodic succession of light and darkness provided the base for relative timing of biological processes over the 24 hours of a day. Because energy supply is the limiting parameter for survival, a system for optimal timing of energy expenditure and uptake developed. The mechanism of this system took the shape of a cycle reflecting the recurrence of sunrise and sunset, and is termed a «circadian clock» – a clock with a period of about one day (latin: *circa diem*). The internalization of environmental time within the organism not only allows organization of biological processes along the 24-hour time scale but also prediction of recurring events, such as availability of food and emergence of predators. The most compelling demonstration of the circadian clock's utility has been made by using cyanobacterial strains with different clock properties growing in competition with each other. Strains with a functioning circadian clock defeat clock-disrupted strains in rhythmic environments, however, this competitive advantage disappears in constant environments. The strains compete most effectively in a rhythmic environment when the frequency of their internal biological oscillator is similar to the environmental cycle (Ouyang et al., 1998; Woelfle et al., 2004). That this is also valid for multicellular eukaryotes has been demonstrated recently in studies using the plant *Arabidopsis thaliana*. Comparing wild type with long- and short-circadian period mutants indicates an advantage of matching the circadian period to the external light-dark cycle. Hence, wild type plants contain more chlorophyll, fix more carbon,

grow faster, and survive better than plants with circadian periods differing from their environment (Dodd et al., 2005).

The underlying principle of circadian clocks is successive gene activation in form of a cycle: the initial gene activation is regulated by the last one in the sequence, making up an auto-regulatory feedback loop for which one cycle takes about 24 hours. This principle is illustrated in figure 1. Positive elements activate the expression of negative elements, which in turn stop the activity of the positive elements. This system moves away from equilibrium before returning and hence, perpetual cycling is the consequence. Although the genes involved in this mechanism can differ in various organisms the principle illustrated in figure 1 is common to all of them (reviewed in Bell-Pedersen et al., 2005; Young and Kay, 2001).

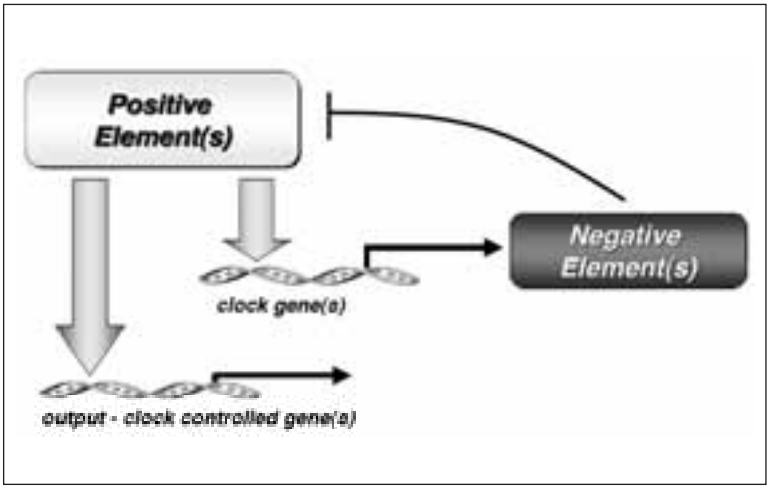

Figure 1:
General mechanism of the circadian clock. Positive elements activate expression of negative elements that inhibit the action of positive elements, thereby establishing an auto-regulatory feedback loop. The positive elements of the clock additionally activate clock-controlled genes transmitting time information to the whole organism.

Temporal information coded in this clock mechanism is only of if it is translated into a physiological meaning. This is achieved through coupling of the clock to biological pathways that are themselves composed of sequential gene activation. Connecting rate-limiting steps to the clock submits whole pathways to a circadian rhythm and hence, they become hands of the clock. This is also termed the clock's output (see figures 1 and 2).

The earth's orbit around the sun leads to seasons that manifest themselves, besides the temperature changes, in an altered length of a day's light period. To adapt to these changes the circadian clock is connected to mechanisms that allow it to stay in tune with nature. Sensory organs communicate environmental time information *via* signaling pathways to the clock, thereby synchronizing the internal circadian oscillators with the environment. The existence of such an input pathway in the circadian system (figure 2) is the reason why humans can adapt to different time zones and overcome jet lag.

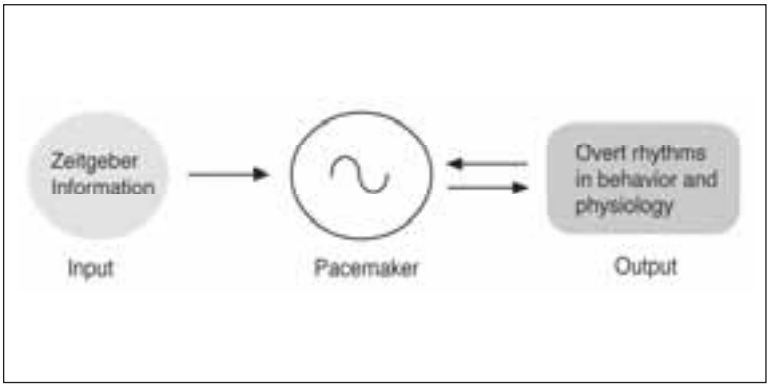

Figure 2:
Schematic diagram of the circadian system. It is composed of the input pathway, the pacemaker or clock and the output pathway. Note that the physiological state of the organism can influence the clock pacemaker to establish a crosstalk between clock pacemaker and output targets.

Figure 2 illustrates the exquisite position of the clock pacemaker in the circadian system. It synchronizes the rhythms in physiology with the environmental diurnal rhythm and hence is not only a metronome but also an integrator of both environmental and body signals. In unicellular and light permissive animals each cell has a circadian oscillator with its own photoreceptors that communicate with the clock to set its phase. This is different in opaque multi-cellular organisms such as mammals. Although each cell in the different organs contains a clock (Yamazaki et al., 2000; Yoo et al., 2004) not every cell of the body can be reached by light. Hence, a hierarchical organization of the individual clocks is necessary (reviewed in Hirota and Fukada, 2004). In mammals the retina and the master pacemaker, located in the *suprachiasmatic nuclei* (SCN) (see figure 3A), are the only known cells to be entrained by light. Consequently, the clocks in the peripheral organs, such as kidney and liver, must be synchronized by neuronal or humoral signals from the SCN. Diffusible factors from the SCN that have the potential to synchronize clocks in different structures of the brain are TGFα and prokineticin 2 (Cheng et al., 2002; Kramer et al., 2001). However, these factors seem not to be the main synchronizers of peripheral tissues because their receptors are lacking in most peripheral organs. Serum inducibility of clock genes in fibroblasts (Balsalobre et al., 1998) suggests that blood-born factors stimulate signal transduction pathways that influence the mammalian molecular oscillator in cells of peripheral tissues (figure 3A). Glucocorticoids might play an important role in this regard, because they can reset the circadian clock by changing *Per* gene expression (Balsalobre et al., 2000) and the hypophyseal-adrenal axis is regulating their expression (reviewed in Buijs and Kalsbeek, 2001). A number of other factors affect *Per* gene expression and clock phase, including interleukin-6 (Motzkus et al., 2002) and retinoic acid (McNamara et al., 2001).

To study the mammalian circadian transcriptional output, several laboratories have applied the systems-biology tool of transcriptional profiling (Panda et al., 2002; Ueda et al., 2002). Analysis of rhythmic genes in liver revealed their principal role in regulating metabolism, whereas genes cycling in the SCN are primarily involved in signaling and neurosecretion. However, the network topology of circadian trans-

criptional output remains elusive. It seems that three promoter elements are important for circadian regulation: E-boxes, targets of the CLOCK/BMAL1 complex, D-boxes, targets of DBP/E4BP4, and REV-ERBα/ROR-regulatory elements (RREs) (figure 3B). Combinations of these three elements in promoters allow generation of different phases and amplitudes of circadian transcription with the E-box element playing the critical role (Ueda et al., 2005).

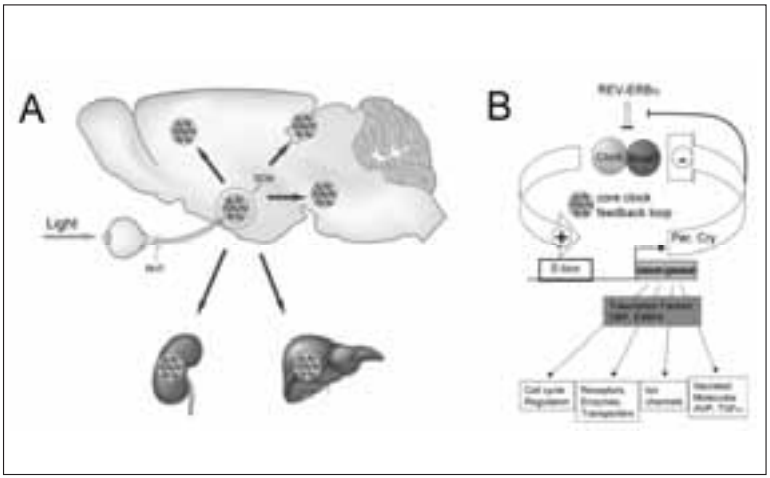

Figure 3:
The circadian system and clock mechanism in mammals. A) Hierarchical organization of the circadian system. Light activates specific photoreceptors in the retina from where the signal is transmitted via the retinohypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN), the master pacemaker. The SCN emits signals to synchronize the clocks (circles with waved lines) in other brain structures and in peripheral organs such as the kidney and the liver. B) Clock mechanism in mammals. The transcriptional activators CLOCK and BMAL1 dimerize and promote transcription of Period (Per) and Cryptochrome (Cry) genes by binding to E-box elements present in their promoters, constituting the positive limb of the feedback mechanism. PER and CRY proteins are thought to inhibit the CLOCK/BMAL1 complex, thereby closing the loop (negative limb). REV-ERBα negatively regulates the Clock and Bmal1 genes and is influenced by PER and CRY. Additionally, protein kinases have important roles in the modulation of the activities of clock components. The positive limb of the loop can also activate output genes either directly (e.g. Avp) or indirectly via the regulation of other transcription factors such as DBP (reviewed in Reppert and Weaver, 2002).

The circadian timing system of mammals influences most physiological activities, including sleep and wakefulness, body temperature, intestinal peristaltics, hepatic activity, cardiovascular activity and precision of the sensory system (reviewed in Schibler et al., 2003). As illustrated in figure 3 these systems depend on the SCN, which receives photic information from classical rod and cone photoreceptors (Freedman et al., 1999; Ruby et al., 2002) as well as from melanopsin containing ganglion cells of the retina (Hattar et al., 2002; Ruby et al., 2002). This information is transmitted as electrical signals *via* the RHT (figure 3A). The neurotransmitters glutamate and pituitary adenylate cyclase-activating peptide released at the RHT synapses contacting the SCN, trigger the influx of calcium which results in an activation of several protein kinases (protein kinase A, PKA; protein kinase C, PKC; protein kinase G, PKG, cGK; mitogen-activated protein kinase, MAPK) (reviewed in Hirota and Fukada, 2004). This leads, besides the stimulation of immediate-early genes, to an activation of *Per1* and *Per2* genes, and the photic regulation of PER protein accumulation may play an essential role in tuning the circadian clock to daylight (Albrecht et al., 2001; Steinlechner et al., 2002). Support for this view comes from the finding that, in humans, a mutation in the casein kinase I binding domain of the *Per2* gene leads to hypophosphorylation and is associated with familial advanced sleep phase syndrome (FASPS) (Toh et al., 2001). This posttranslational regulation has a prominent effect on the clock and is further illustrated by the observation that a mutation in the casein kinase I delta can cause FASPS probably by affecting phosphorylation of clock components (Xu et al., 2005).

Single SCN neurons cultured *in vitro* display circadian rhythms in firing frequency and therefore, contain autonomous oscillators (Liu et al., 1997). Interestingly, the period length varies between the cells, causing desynchronization between them after prolonged time in culture. Therefore, in the intact organism the SCN neurons must be coupled. Coordinated activity of SCN neurons seems to be critically regulated by electrical synapses (Long et al., 2005) and vasoactive intestinal polypeptide (Aton et al., 2005). This coupling appears to be strong enough to maintain a circadian rhythm in rodents even when they are kept in constant darkness for months or even years. In contrast,

when exposed to constant light, the animals become behaviorally arrhythmic with time. This is not due to disruption of the circadian firing rhythm but is the result of desynchronization of clock neurons (Ohta et al., 2005).

The findings described above highlight the importance of synchronization. Internal body time needs to be aligned with external environmental time, and within the body, the clocks in different organs and within organs need proper orchestration if our body is to withstand predictable natural forces and work in an optimized fashion. However, in our 24-hour society shift work and transmeridian flights are a serious challenge for our circadian system, and through its coupling to physiological pathways also for our health. These challenges are relatively well handled in younger organisms, but with age, associated problems like sleep disturbances, digestive and cardiovascular problems, mental illness and alcohol abuse become more apparent. The reason for this is that circadian organization changes with age (Valentinuzzi et al., 1997; Yamazaki et al., 2002) and results in decrease of amplitude and fragmentation of the rest-activity cycle as well as in a reduced sensitivity to the phase-shifting effects of light (Valentinuzzi et al., 1997). These age-related phenotypes can be mimicked by alterations in the clock mechanism (Oster et al., 2003). A mutation in the Clock gene disrupts estrous cyclicity and maintenance of pregnancy (Miller et al., 2004) probably caused by an uncoupling of prolactin regulation from the clock (Leclerc and Boockfor, 2005). The findings described above illustrate a potential relation between the circadian system and the aging process.

A very prominent age-related process is the development of cancer. Because a defective clock seems to accelerate aging, it is not surprising to find that mice with a defective clock are more prone to develop cancer. The *Per2* gene plays a role in tumor suppression and in DNA damage response by regulating the temporal expression of genes involved in cell cycle regulation, such as *c-Myc, Cyclin D1, Cyclin A* and *Mdm-2* (Fu et al., 2002). In the regenerating liver of mice it has been shown that the circadian clock directly controls the expression of cell cycle-related genes, such as *wee1*, that in turn modulate the expres-

sion of active Cyclin B1-Cdc2 kinase, a key regulator of mitosis (Matsuo et al., 2003). Interestingly, circadian gene expression in fibroblasts continues during the cell division cycle and daughter cells have the same phase as their mother cell. It seems that the circadian oscillator gates cytokinesis to define time windows, and mitosis elicits phase shifts in circadian cycles (Nagoshi et al., 2004). However, the interaction between the circadian clock and the cell cycle appears to be reciprocal. In breast cancer the expression of *Per* genes is deregulated (Chen et al., 2005) and many tumor cells have lost circadian rhythmicity and daytime-dependent cell cycle progression (reviewed in Canaple et al., 2003). This difference between healthy and cancer cells might be exploited by delivering antiproliferative drugs at times when they are least toxic to normal cells. Encouraging results are now being obtained with the first attempts of using chronotherapy (reviewed in Mormont and Levi, 2003) and this might lead the way for more efficient and less harmful treatments of cancer patients. A prerequisite for the success of chronotherapy is the determination of an individual's body time. Reaching this goal is not an easy task, because of tissue sampling at multiple time points. However, a microarray-based method has been developed to determine individual body time in mice, from tissue harvested at a single time point (Ueda et al., 2004). How applicable this method is for humans, remains to be seen (reviewed in Albrecht, 2004) but it is a first step in the direction of individualized medicine, which could improve medical treatment.

Peripheral clocks are not exclusively influenced by the SCN in the brain. Feeding cycles can act directly on the clock in peripheral organs and uncouple their clock phase from the SCN (Damiola et al., 2000). This suggests that these clocks have an important role in the processing of nutrients and energy homeostasis. Several studies using transcriptome profiling support this view, showing that many cyclically expressed liver genes perform functions related to metabolism and detoxification (Panda et al., 2002; Storch et al., 2002). The connection between metabolism and the clock is particularly intriguing in view of the finding that DNA binding of Clock protein is regulated by the redox state of NAD cofactors (Rutter et al., 2001). This highlights the regulatory potential of metabolism on the clock, however, recent studies also

indicate that alterations in the circadian clock mechanism may change metabolism. Mice with mutations in the clock genes *Bmal1* and *Clock* show no diurnal variations in glucose and triglycerides, and gluconeogenesis is altered in these mutants, indicating that the circadian clock is involved in glucose homeostasis (Rudic et al., 2004). Additionally, *Clock* mutant mice become obese and develop metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia and hypoinsulinemia (Turek et al., 2005). These findings lead to the speculation that in our society, due to irregular life style, our clock might become derailed, promoting abnormal eating habits and obesity. Indeed, studies with human volunteers indicate that short sleep duration is associated with reduced leptin levels, elevated ghrelin and an increased body mass index (Taheri et al., 2004).

Deregulation of metabolism has far reaching consequences. It not only leads to an alteration in body mass index but can also affect mental state. The amounts of glutamate, dopamine, serotonin and other neurotransmitters seem to be, at least in part, under the influence of the circadian clock via regulation of DBP or other unknown genes. Lack of three proline and acidic amino acid rich basic leucine zipper (PAR bZip) transcription factors, DBP (albumin D-site binding protein, Fig. 3B), HLF (hepatic leukemia factor) and TEF (thyrotroph embryonic factor) results in epilepsy. This is due to an alteration in pyridoxal kinase (Pdxk), which converts vitamin B6 derivatives into pyridoxal phosphate (PLP), the coenzyme of many enzymes involved in amino acid and neurotransmitter metabolism. In mice a lack of these PAR bZip factors results in decreased PLP, serotonin and dopamine levels in the brain leading to epilepsy (Gachon et al., 2004a). Another neurotransmitter, glutamate, is also affected in mice with an altered clock. Normally, the excitatory amino acid transporter 1 (EAAT1), which transports glutamate from the synaptic cleft into astrocytes, is expressed in a circadian fashion. A mutation in the *Per2* gene reduces the expression of EAAT1, and thus, glutamate levels in the synaptic cleft rise. However, the amount of glutamate does not reach toxic levels but they are sufficiently high to alter the behavior of these animals, e. g. adjustment of the clock to the day-night cycle (Albrecht et al., 2001) or alcohol consumption (Spanagel et al., 2005).

The first evidence for the involvement of the circadian clock in addictive processes has come from the observation that fruit flies mutant in clock genes do not sensitize to cocaine through regulation of tyrosine decarboxylase (Andretic et al., 1999). Similar observations have been made in mice (Abarca et al., 2002; McClung et al., 2005), suggesting that clock genes are involved in common modulator mechanisms of drug abuse-related behaviors (Yuferov et al., 2003). Interestingly, drugs modulate clock genes, as demonstrated by methamphetamine injection causing an increase of *Per* gene expression in the caudate putamen of the mouse (Nikaido et al., 2001). Metamphetamine treatment of rats alters the circadian expression rhythms of clock genes in the caudate putamen and the parietal cortex, and desynchronizes them from the SCN rhythms (Masubuchi et al., 2000). Drug action seems to involve common signal transduction pathways that are also part of the resetting mechanism of the circadian clock. For example, serotonin receptor agonists phase shift the circadian clock through an increase in cAMP production (Sprouse et al., 2004) and the drug Ecstasy (3,4 – methylenedioxymethamphetamine) alters this response (Biello and Dafters, 2001). Similarly, opioids affect the circadian system, suggesting a modulatory role for the clock in pain sensation (Vansteensel et al., 2005).

During episodes of depression the balance of a variety of neurotransmitters is disturbed. This balance might be tuned by the circadian clock as evidenced by the findings described above, and light probably plays an important role for orchestrating the temporal pattern of neurotransmitters for synchronization with the environment. An involvement of circadian clock related polymorphisms both in seasonal affective disorder (SAD) and in diurnal preference was found (Johansson et al., 2003), supporting the hypothesis of a link between circadian rhythms and seasonal depression. Whether a defective circadian clock plays a role in bipolar disorder (BD) is not clear, although there is evidence for a significant genetic etiology. However, gene-mapping efforts have been hampered by the complex mode of inheritance and the likelihood of multiple genes involved with small contribution. Because the circadian clock has a wide regulatory potential and extensive disruption in circadian function is known to occur among patients with BD during

relapse, it is plausible that circadian dysfunction underlies pathogenesis of BD (reviewed in Mansour et al., 2005).

The spatial and temporal distribution of electrical activity is a key modulator of the constructive and destructive processes that determine neuronal form and sculpt the pattern of neural circuitry. Therefore, one can speculate on the involvement of the circadian clock in the process of constant rearrangement. This may involve structural changes or modulation of the efficacy of synapses, both of which alter the functional properties of neural networks. This plasticity is crucial for numerous brain functions, most notably learning and memory, and may also explain addiction-related behaviors (see above). Circadian modulation of learning and memory has been investigated with varied results. In *Aplysia* long-term sensitization seems to be modulated in a circadian manner (Fernandez et al., 2003) and the clock gene *period* plays a key role in long-term memory formation in *Drosophila* (Sakai et al., 2004). Learning experiments in rats using the Morris water maze task demonstrated that circadian phase has an effect on learning performance (Valentinuzzi et al., 2004). However, mice mutant in the *Per1* or *Per2* gene do not differ in comparison to wild type animals in hippocampus-dependent learning (Zueger et al., 2005). Because these experiments have only used a limited set of tests under diurnal conditions more detailed studies are warranted in mammals.

The physiological and mental state of mammals alters throughout the day as manifested in the sleep-wake cycle. At the level of neurons this is paralleled by the steady depolarization of these cells during the day. Also, glucose metabolism in the brain is higher during wakefulness (Maquet et al., 2000) and the main astroglial glucose transporter *GLUT1* as well as the mitochondrial genes involved in oxidative phosphorylation are expressed during wakefulness. Similarly, genes of the glutamate-glutamine cycle (*glutamine synthase and glutaminase*) and regulatory genes for clustering glutamatergic receptors (*Homer/ Vesl, Narp*) are also expressed predominantly during wakefulness (Cirelli et al., 2004). While wakefulness has been associated with memory acquisition, sleep represents a favorable time for memory consolidation (Stickgold, 2001; Stickgold et al., 2001; Walker et al., 2002).

Many key components of the translational machinery are expressed at higher levels during sleep (Cirelli et al., 2004). Taken together these observations indicate that though sleep is a state of behavioral inactivity it is associated with the expression of many genes in the brain, and sleep and wakefulness favor different cellular processes. Wakefulness related transcripts may aid the brain in facing high energy demand, high synaptic excitatory transmission, high transcriptional activity and in the need for synaptic potentiation in the acquisition of new information as well as the cellular stress that may derive from one or more of these processes. Sleep, on the other hand, favors protein synthesis and complimentary aspects of neural plasticity such as synaptic depression. Interestingly, a mutation in a voltage-dependent potassium channel controlling membrane repolarization and transmitter release has been identified to be causal to short sleep in *Drosophila*. This mutation in the Shaker gene affects not only sleep duration but also leads to a reduced lifespan (Cirelli et al., 2005a).

Sleep is composed of homeostatic and circadian processes (Borbely, 1982; Borbely and Achermann, 1999) and hence the circadian clock is part of sleep. For example, mutations in *Per1* and *Per2* genes in the mouse influence allocation of sleep in the 24 hour day but these mutations do not affect the EEG slow-wave activity (Kopp et al., 2002). In contrast, *Cry1/2* double mutant mice exhibit high non-REM sleep (Wisor et al., 2002), highlighting a role of these clock components in homeostatic aspects of sleep. Microarray analysis of gene expression in *Drosophila* revealed that many wakefulness-related and sleep-related transcripts are modulated by time of day, suggesting an interaction at the molecular level between circadian and homeostatic mechanisms of sleep regulation (Cirelli et al., 2005b).

Perspectives

In the past few years many of the molecular components of the circadian clock have been identified in several organisms. This led to a general understanding of the clock mechanism and its potential to influence physiological processes. In the future the relationship

between metabolism and the circadian clock will receive more attention. This is because mitochondrial function depends partially on nuclear transcription of its enzyme complexes in the oxidative chain, which is directly related to generating the organism's energy currency ATP.

The clock mechanism itself will also be completed with satellite feedback loops, and inconsistencies in the current model will be addressed (see Gachon et al., 2004b). The regulation of clock components at the transcriptional and post-transcriptional levels will be major issues. Is regulation at the RNA level occurring in mammals as it is in other phyla (Kramer et al., 2003)? The determination of the three-dimensional structure of clock components (Yildiz et al., 2005) will boost our understanding in how these molecules interact with each other and with other molecules. This will help to decipher the precise function of the mammalian *Period* genes and answer the question whether they are co-activators or co-repressors of transcriptional activation.

At the cellular level future work will concentrate on the mechanisms by which cells couple and synchronize their circadian clock in a tissue. This will be greatly facilitated by the use of reporter constructs (Nagoshi et al., 2004), allowing direct monitoring of circadian clock activity in live cells. Such an *in vitro* system will be of great help to apply interfering RNA technology for evaluating the contribution of novel candidate clock genes.

At the physiological level one of the main challenges will be to unravel the role of the clock in mental capabilities. How does the clock influence the development of depression? Does it modulate pain sensation? What are the environmental contributions in altering the clock phase, causing aberrant mental states? Are there causative relationships between the clock, lifestyle and neurodegeneration causing Alzheimer's or Parkinson disease?

Individually tailored medicine is still a dream but with our knowledge on the circadian clock and clock-regulated processes improvement in efficiency of pharmacological agents will be possible. Individually

adapted treatment due to chronotyping of patients (Brown et al., 2005) will allow us in the future to improve timing of medical treatment, and thus, to reduce harmful side effects. Indeed, it appears that, for our health, timing is everything.

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