A cellular model for human daily behaviour

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SUMMARY

All the biochemical, physiological or behavioural processes whose period is about 24 hours possess a circadian rhythm. Circadian rhythms are very well conserved in beings of different levels of complexity since they provide evolutionary advantages by anticipating external changes. Indeed, circadian rhythms control almost all aspects of human daily behaviour and physiology, from the sleep-wake cycle to memory and alertness. The disruption of circadian rhythms leads to many diseases, including depression, cancer and metabolic syndromes. Understanding the mechanisms and function of circadian rhythms is essential to a better treatment of circadian pathologies and to avoid the exacerbation of co-morbidities such as depression, metabolic syndromes and vascular diseases. However, the study of human circadian rhythms in subjects *in vivo* is expensive, very time consuming and may involve the use of invading devices. In complex animals like mammals circadian rhythms are organized in a hierarchic fashion: suprachiasmatic nucleus (SCN) is master clock and governs the circadian rhythms of all peripheral oscillators, virtually all the other cells of the body. SCN and peripheral oscillators share the same circadian molecular machinery. It is thus possible to use peripheral oscillator as model to study molecular mechanisms of circadian rhythms.

The aim of the thesis was to establish the use of human primary skin fibroblasts as a valuable model to study different aspects of human circadian rhythms. To visualize in real-time cellular circadian rhythms, fibroblasts were infected with a lentivirus coding for the circadian reporter firefly luciferase under a clock gene promoter (Bmal1). After the synchronization of circadian rhythms, the measurement of the light emitted by the cells gives a representation of fibroblast circadian oscillations. Several circadian parameters can be analysed by the measurement of fibroblast bioluminescence, such as phase (e.g. the phase of sleep-wake cycle that is different between the “larks”-early chronotypes - and “owls”-late chronotypes), period length (the time spent for one complete cycle), amplitude (the magnitude of change in an oscillating variable) and damping rate (e.g. progressive reduction of the circadian amplitude due to desynchronization).

This thesis was designed to (**1**) validate the human skin fibroblast model, ascertaining that this *in vitro* model parallels *in vivo* human circadian parameters, and (**2**) to study human age-related circadian impairments. Moreover, skin fibroblasts were used (**3**) to investigate the role of melatonin as zeitgeber on peripheral oscillators. Melatonin is secreted in a

circadian fashion and was demonstrated to influence SCN circadian rhythms; however, melatonin functions on peripheral cells are still unclear.

(**1**) The protocols to study human physiological or behavioural circadian rhythms - which are normally governed by the SCN of the brain hypothalamus - involve extensive subject observation under special controlled conditions. Since SCN and peripheral oscillators share the same circadian molecular machinery, we have measured human *in vivo* period length and compared with skin fibroblast period length isolated from the same subjects. *In vivo* period length was determined by the analyses of melatonin or melatonin metabolite content in two groups of sighted subjects and one group of blind participants, each using different protocols. In all the three groups of subjects a very good correlation was found between the *in vivo* and the *in vitro* period length. Interestingly, although the *in vivo* period obtained from the blind group was longer than the *in vivo* period obtained from the sighted groups, the *in vitro* period length from the three groups of subjects was similar, revealing that human skin fibroblasts are insensitive to the after-effects caused by light. This finding reveals the importance of skin cells as a tool to study human circadian rhythms: the factors that determine plasticity in human period do not interfere with fibroblast period length. In summary, human circadian period can be approximated by measurement in fibroblasts.

(**2**) Ageing is a process that leads to impairments of many physiological processes, including circadian rhythms: the sleep-wake cycle of elder persons is disrupted and animal studies revealed an earlier phase of gene expression in some peripheral organs and a shorter SCN period length. Moreover, young subjects have a later chronotype than older subjects. To investigate the molecular mechanisms which lead to circadian changes that occur in humans during ageing, fibroblast circadian parameters from a group of young and older donors were characterized. No differences in amplitude, phase and period length were found between cells from the two groups. These results revealed that, during ageing, the molecular components of the skin fibroblast oscillators do not change *per se*; however, since age-related circadian differences can be observed in peripheral organs, hormone levels, human age-related alterations might be caused by one or more circulating factors. In support of this hypothesis, human fibroblasts in the presence of sera from older donors (instead of foetal bovine serum used under standard conditions) showed a reduced period length and a shorter phase of entrainment compared to the same cells measured in the presence of sera from young donors. These differences are likely due to one or more

thermolabile substances, since heat-inactivation of sera from older donors almost undid the reduction of the circadian period length. In summary these results suggest that during ageing the molecular machinery of peripheral circadian clocks does not change *per se*, but some age-related circadian changes observed *in vivo* might be caused by circulating molecules.

(**3**) Melatonin was previously shown to regulate the SCN firing rate and to entrain the sleep-wake cycle of most mammals and humans. The circadian presence of melatonin is well conserved in all biological fluids, suggesting that melatonin may be one of the molecules that the master clock uses to synchronize peripheral oscillators. This hypothesis was tested in damped fibroblasts, using a wide range of concentrations of melatonin to restore the amplitude of the rhythms. However, no increase of amplitude or phase shift of the rhythms was observed after treating cells with melatonin. Moreover, the application of the hormone to newly synchronized oscillators decreased their bioluminescence. Although these experiments demonstrated that melatonin does not directly synchronize human fibroblasts, they cannot exclude indirect effects of melatonin to entrain cellular circadian rhythms: indeed, decreasing the amplitude of a rhythm may render it more sensitive to phase shifting, for example by substances other than melatonin. In summary, the experiments demonstrated that melatonin does not play a direct role as peripheral oscillator zeitgeber.

In conclusion, the studies of the present thesis succeeded in revealing three primary findings: first, fibroblast circadian rhythms parallel human circadian physiology, such as circadian period length. Second, apparently, during ageing the molecular components of peripheral circadian clocks in skin fibroblasts do not change per se, but some age-related circadian changes observed *in vivo* might be caused by one or more heat-sensitive substances present in the blood of older subjects. Finally, melatonin does not possess direct synchronizing properties on peripheral oscillators like fibroblasts, although it may render the cells more sensitive to further synchronizing signals. In total, the present thesis revealed that primary human skin fibroblasts are an easily accessible, cheap and reliable model to enlighten our understanding of human circadian mechanisms.

1. INTRODUCTION

1.1 CIRCADIAN RHYTHMS

Circadian oscillations, which occur with a period of about 24 hours, play a key physiological role in the adaptation of living organisms to the alternation of day and night by anticipating the external changes. For example, the circadian clock of plants allows them to produce photo-system I and II components already before sunrise, so that photosynthesis can commence as soon as light energy is available [1]. On a similar note, a nocturnal rodent possessing a circadian timekeeper can anticipate dusk in his underground habitat and does not have to forage periodically to examine whether sunset is approaching. Such anticipation may considerably reduce exposure to day-active predators and thus provide a selective advantage. In liver the accumulation of about 5–10% of all messenger ribonucleic acid (mRNAs) undergoes a daily oscillation [2-5]. The identification of these cyclic transcripts has led to the conclusion that a large fraction of circadian genes are involved in metabolism. Thus, many enzymes playing important roles in the utilization or degradation of proteins, lipids, carbohydrates and xenobiotic substances appear to be synthesized in a rhythmic fashion. Reactions catalised by the cytochrome p450 (CYP), enzymes involved in the hepatic detoxification of xenobiotic substances, can generate reactive oxygen species (ROS) [6]. CYP expression is mostly circadian, with peak expression during the absorptive phase [7-8], probably to schedule the production of harmful side molecules in a time window where also the scavengers are produced. Within the circulatory system, heartbeat and blood pressure vary in circadian fashion and rhythmic expression of genes involved in fibrinolysis are believed to account for the prevalence of infarctus at morning hours [9]. Also daily variations in mood and alertness are circadian and might be explained by circadian expression of ion channels, neuronal receptors and hormones [10-11].

1.1.1 Clock genes and circadian machinery

The molecular mechanisms that regulate circadian rhythm are present in several levels of organism complexity, from bacteria to plants to animals. That genes control circadian rhythms was first established in the fruit fly after the demonstration that certain point mutations in *Drosophila melanogaster* cause altered circadian rhythms [12]. The mutated gene, *period* (*per*), was subsequently isolated [13] and was found to be expressed in a circadian pattern [14]. This finding and similar data from bacteria, fungi, and plants, has led

to the general idea that periodically expressed genes constitute the physiological basis of circadian clocks in all living organisms [15-17]. The maintenance of a rhythm of about 24 hours long is made possible by the so called transcriptional-translational feedback loop model. In this model it can be possible distinguish positive components (basically transcription factors) and negative components (inhibitors of the transcription factors).
Some of the proteins that control the circadian process in mammals are related to those found in fruit flies [18-19]. The locomotor activity of *Drosophila* is circadian. Indeed, under light-dark conditions flies show two peaks of activity: in the morning, around the time of lights-on and in the evening, around the time of lights-off. *Drosophila* activity increases in advance of both dark-to-light and light-to-dak transition, a phenomenon termed anticipation. The behaviour of flies is also sensitive to masking, by changing their behaviour in relation to unexpected changes in lighting. When flies are located in constant darkness their activity occurs at approximately the same time each day corresponding roughly to the evening activity peak and reflecting circadian clock function with a near-24 hours period [20]. The circadian behaviour in *Drosophila* is driven by clock genes: *per* and *timeless* (*tim*) genes are transcriptional activated by the binding of dCLOCK (dCLK) and CYCLE (CYC, also called dBMAL-1) transcription factors to the E-boxes present in their genes. After dusk *per* and *tim* levels peak. In the cytoplasm PER and TIM form the complex PER/TIM protein. The rate of appearance of this complex is delayed by the activity of DOUBLE-TIME (DBT), a *Casein kinase* (CK) $I\varepsilon$ homologue, which helps create a 4–6 hours lag between the peak levels of per and tim RNAs and proteins. PER/TIM complex enter into the nucleus at midnight. TIM is degraded and PER is liberated as a nuclear monomer that is progressively phosphorylated and subsequently degraded after dawn [21].

Similarly (Fig. 1), the mammalian players of the negative key components of this genetic circuitry are the four genes encoding *Cryptochrome* 1 (*Cry1*), *Cryptochrome* 2 (*Cry2*), *Period* 1 (*Per1*) and *Period* 2 (*Per2*). These genes are regulated by the two PER-ARNT-SIM (PAS) domain basic helix-loop-helix transcription factors CLOCK and BMAL1, the positive key components of the circadian oscillator. CLOCK and BMAL1 form a heterodimer that binds E-Boxes sequences present in *Per* and *Cry* promoters. The binding to E-boxes by CLOCK-BMAL1 complex activates the transcription of *Per* and *Cry* genes. PER and CRY proteins form heteropolymeric complexes of unknown stoichiometry and once these complexes have reached a critical concentration in the cell nucleus, they

interact with the CLOCK-BMAL1 heterodimer inhibiting CLOCK-BMAL1 dimerization and their binding to E-boxes, leading to a block of *per* and *cry* expression. As a consequence *cry* and *per* mRNAs and proteins decrease in concentration and once the nuclear levels of the CRY–PER complexes are insufficient for auto-repression, a new cycle of *Per* and *Cry* transcription can start [22-23] (see also 6.1 Appendix 1). Many additional components contribute to the robustness of this molecular clockwork circuitry. For example, the orphan nuclear receptor and repressor REV-ERBα interconnects circadian transcription of the positive and negative “limbs” of the oscillator. REV-ERBα transcription is activated by CLOCK-BMAL1 complex through the binding to E-box sequences present in its promoter, resulting in its circadian accumulation. REV-ERBα protein leads to periodic repression of *Bmal1* and *Clock* transcription. This repression leads to a rhythmic expression of *bmal1* and (to a lesser extent) *clock* mRNA in antiphase with *Rev-Erbα* expression [24]. Posttranslational mechanisms such as protein phosphorylation also play important roles in generating oscillations of approximately 24 h. For example, *CK1ε*, initially identified as an essential *Drosophila* clock component [25], phosphorylates PER, CRY, and BMAL1 proteins [26-28]. CK1δ, a close paralog of CK1ε, has also been found to be associated with PER–CRY complexes and may therefore perform a similar function as CK1ε [29]. Hypophosphorylated PER proteins have a higher metabolic stability than their hyperphosphorylated counterparts and this may lead to an increased accumulation of PER proteins.

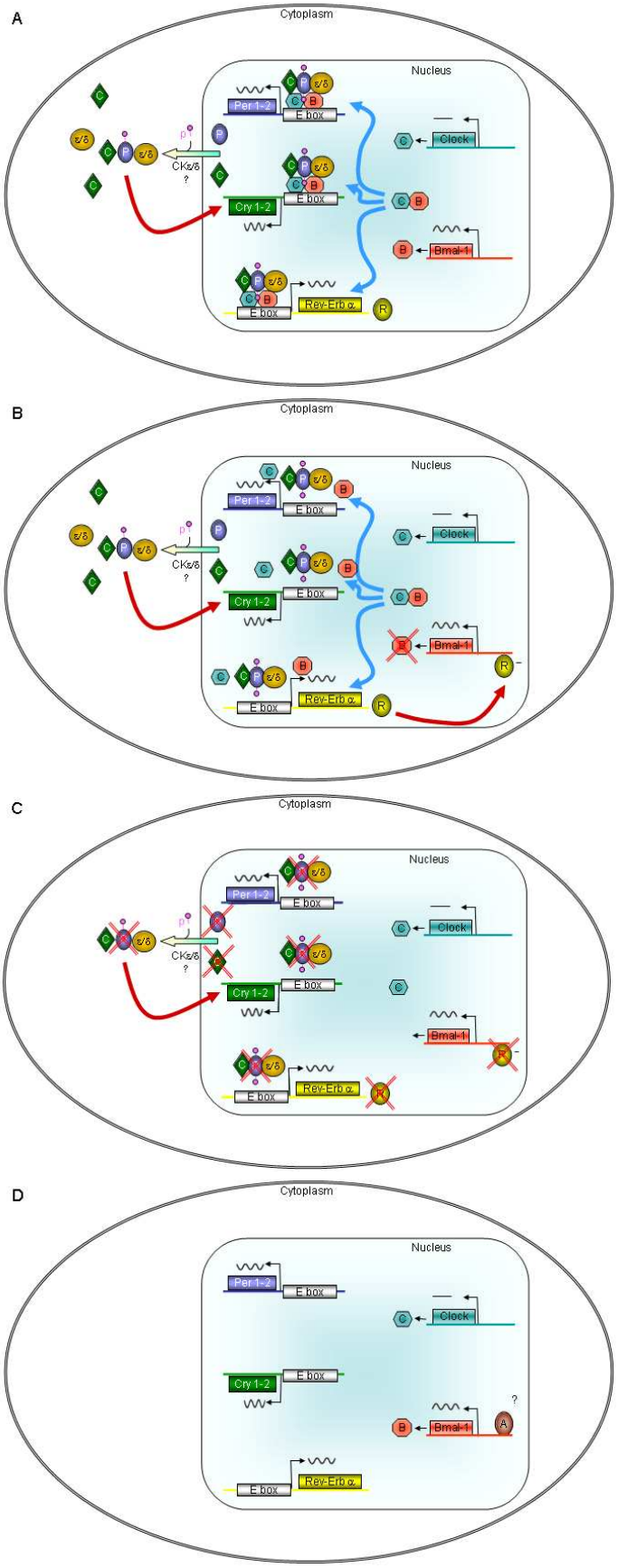

Fig. 1. Molecular circadian clock machinery. **(A)** The positive key components of the negative feedback loop, BMAL1 (B, red octagon) and CLOCK (C, green esagon), dimerize, bind to specific promoter sequences, E-Box, and activate the transcription of the negative elements *Per* (P, violet ellipse), *Cry* (C, green rhomb) and *Rev-Erb*α(R, yellow ellipse). In the cytoplasm PER and CRY protein form a complex of unknown stochiometry with CKIε/CKIδ (ε/δ, orange ellipse), that enter in the nucleus, binds the heterodimer BMAL1/CLOCK. **(B)** The complex PER/CRY/CKI inhibits BMAL1/CLOCK binding to E-Box sequences and the heterodimerization of BMAL1 with CLOCK. At the same time REV-ERBα inhibits the transcription of *Bmal1*, decreasing BMAL1 content in the cell. **(C)** As a consequence, also the negative key components CRY and PER and REV-ERBα decrease their concentration in the cell. **(D)** Once the presence of the negative components of the feedback is reduced, BMAL1 protein is produced again, probably through an activator (A, brown ellipse). (Adapted from [23]).

Circadian rhythms are conserved also in fungi. The widely used model to study fungal circadian rhythms is *Neurospora crassa*. In *Neurospora* the most obvious circadian output is the formation of a mass of spores, called conidia. Conidiation is a time-consuming process that is initiated after dusk and reaches a peak in the late (subjective) night. Conidiation is driven by clock genes machinery: by late subjective night, *frequency* (*frq)* mRNA levels are low. WHITE COLLAR (WC)1 and WC-2 act together as a transcription factor, the white collar complex (WCC), by binding to a specific sequence (the Clock Box [30]) on the *Frq* promoter in order to drive transcription. FRQ protein appears by between late night and morning. By early subjective morning FRQ proteins are synthesized [31] and they dimerize [32] and interact with the RNA helicase FRH [33] before entering the nucleus [34]. As soon as FRQ appears, it begins to be phosphorylated by several kinases (i.e. casein kinase I and II). Phosphorylation of FRQ serves to facilitate interaction of FRQ with the ubiquitin ligase FWD-1 that targets FRQ for turnover in the nucleosome [35]. In the nucleus, specific interactions occur between the FRQ–FRH complex and the WCC [36-38] that block the activity of the WCC [30], probably by affecting the phosphorylation status of either or both WC-1 and WC-2. As a result, by mid-subjective day WCC activity declines to its lowest level [30, 39] and this dampened expression of *frq* RNA also causes its level to begin to fall.

The simplest organisms known to show circadian rhythms are the photoautotrophic cyanobacteria [40] where a robust circadian cycling has been observed even under constant darkness. In cyanobacteria many processes are under circadian control, including global gene expression: the entire chromosome undergoes daily cycles of topological changes and compaction. In *Synechococcus elongatus* many processes conform optimally to the daily cycle, including photosynthesis, nitrogen fixation, and gene expression [41]. In contrast to eukaryotic clock models the cyanobacterial clock operates independently of transcription and translation processes. Three genes, *KaiA*, *KaiB*, and *KaiC*, play essential roles in the circadian clockwork of a cyanobacterium [40, 42]. Multiple phosphorylation states [43], allosteric rearrangement [44], KaiA sequestration [45], monomer exchange [46-47] or different combinations of them [48-49] have been suggested to serve as basic mechanisms producing synchronized oscillations. In a proposed model KaiC represses the expression of *kaiB* and *kaiC* and thus constitutes a negative feedback loop [40, 50]. KaiA catalyzes the phosphorylation of KaiC [51] and KaiB attenuates the kinase activity of KaiA [52], where KaiA interacts with KaiC mostly in the

KaiC phosphorylation phase and the KaiB activity is most evident in the KaiC dephosphorylation phase [53-54].

1.1.2 Sleep

One of the classical examples of circadian rhythms in animals is the sleep-wake cycle.
Sleep is an active and as yet poorly understood process, during which many physiological and cerebral events occur. In general, the daily sleep/wake cycle is under circadian control, although the urge to sleep appears to be controlled by brain functions that are independent of the circadian system [55]; thus, sleep-wake cycles are regulated by the interplay of a homeostatic mechanism, which regulates sleep depth, and the circadian clock, which regulates timing of sleep.

1.1.2.1 Sleep architecture

When humans and animals fall asleep some characteristic behaviours, such as reduced motor activity, decreased response to stimulation, stereotypic postures (in humans, for example, lying down with eyes closed) and relatively easy reversibility (distinguishing it from coma, hibernation, and estivation) occur. Despite the typical reduction of motor activity sleep is not a static process. Several stages of sleep, coded in 2004 by the American Academy of Sleep Medicine as non-rapid eye movement (NREM) sleep (divided in stage N1-N4) and rapid eye movement (REM) sleep (stage R) [56], come one after the other, normally cycling from NREM sleep and then REM sleep. Human adults usually begin sleep by progressing from stage 1 through stage 4 of NREM sleep. This progression is intermittently interrupted by body movements and partial arousals. After about 70-80 minutes the sleeper usually returns briefly to stage 3 or stage 2 and then enters the first REM phase of the night, which lasts about 5-10 minutes. In humans the length of the cycle from the start of NREM sleep to the end of the first REM phase is about 90-110 minutes. This cycle of NREM and REM sleep is typically repeated four to six times a night. In successive cycles the duration of NREM stages 3 and 4 decreases while the length of REM phases increases. In young adults the largest amount of sleep time (50-60%) is spent in stage 2 NREM sleep; REM phases constitute 20-25% of total sleep time, stages 3 and 4 NREM about 15-20%, and stage 1 NREM about 5%. Sleep stages can be monitored via electromyography, to measure muscle activity, electro-oculography, to measure eye movements and electroencephalography, to measure the collective activity of cortical neurons.

NREM sleep is characterized by low neuronal activity, metabolic rate and brain temperature. In addition, sympathetic outflow decreases and heart rate and blood pressure decline. Conversely, parasympathetic activity increases and then dominates during the NREM phase, as evidenced by constriction of the pupils. Muscle tone and reflexes are intact.

NREM sleep stages are:

Stage 1: this stage represents the transition from wakefulness (low voltage eletroencephalography (EEG) activity, that normally ranges between 10-30 μV and 16-25 Hz) to the onset of sleep (EEG appearance of alpha activity, a sinusoidal activity ranging between 20-40 μV and 10 Hz) and lasts several minutes. In stage 1 and throughout the NREM phase there is some activity of skeletal muscle but no rapid eye movements.

Stage 2: stage 2 is characterized by bursts of sinusoidal waves called *sleep spindles* (12-14 Hz) and high-voltage biphasic waves called K complexes, which occur episodically against a background of continuing low-voltage EEG activity.

Stage 3: during stage 3 NREM sleep delta waves (0.5-2 Hz) in the EEG occur.

Stage 4: in that stage the presence of slow-wave activity dominates the EEG record.

In humans stages 3 and 4 are also called *slow-wave activity sleep.*

REM sleep comes after NREM sleep. In humans REM sleep is characterized by an EEG presenting a low-voltage and mixed-frequency pattern that in some cases resembles those during active wakefulness. Indeed certain neurons (those in the pons, the lateral geniculate nucleus, and the occipital cortex) actually fire in more intense bursts during REM sleep than during wakefulness. These intense bursts of high-voltage firing, called ponto-geniculo-occipital spikes (*PGO spikes*) for brain structures in which the spikes appear most prominently, correlated with the bursts of eye movements in REM sleep. Tipically, during REM sleep, brain temperature and metabolic rate rise, atonia of almost all skeletal muscle occur (with the exceptions of muscles controlling the movements of the eyes, middle ear ossicles, and diaphragm) even if some small phasic twitches may happen. Miosis reflects the high ratio of parasympathetic to sympathetic output to the pupil. Homeostatic mechanisms are attenuated: respiration is relatively unresponsive to changes in blood CO_2 and responses to heat and cold are greatly reduced or even absent. As a result, body temperature drifts toward ambient temperatures.

1.1.2.2 Sleep regulation

The latest proposed model of sleep regulation posits the interaction between two main independent regulatory processes, the circadian process and the homeostatic process [57], that together determine timing, duration and quality of both sleep and wakefulness. Where the circadian system determines the distribution of sleep over the day, the homeostatic process tracks sleep need. Sleep need and the propensity to initiate sleep increase during wakefulness and decrease while asleep. The daily (circadian) sleep–wake alternation also makes the homeostatic process appear as a 24 hours oscillation with the important distinction that this oscillation is imposed or driven by the sleep–wake distribution whereas circadian rhythm is self-sustained. One of the best protocol to study the interaction between these two processes in humans is the forced desynchrony protocol [58]. Trought the use of forced desynchrony protocol it was possible to understand that the circadian system generates a sleep–wake propensity rhythm that is timed to oppose homeostatic changes in sleep drive. This enables us to stay awake and alert throughout the day despite an accumulating need for sleep and asleep during the night despite a waning of sleep need [59]. Slow wave activity (delta power) reflects the sleep need and so it is considered as a measure of sleep homeostasis [60], whereas circadian clock pacemaker firing rate is a measure of the circadian time. From forced desynchrony and constant routine studies it seems that sleep homeostasis and circadian rhythms are two processes that interact in a non-additive and perhaps independent manner in order to create, for instance, human sleep-wake cycle. Indeed, in humans the amplitude of the observed circadian variation in alertness, performance and numerous sleep variables depend on the status of the sleep homeostat [59]. Moreover, several clock genes are implicated in sleep homeostasis, suggesting that the molecular circuitry used to set internal time-of-day might equally be utilized to track and anticipate sleep need [61]. Circadian rhythms and sleep homeostasis are physically interconnected: the circadian pacemaker located in the suprachiasmatic nucleus (SCN) is physically connected to brain areas involved in sleep, such as the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus [62] who project cholinergic input. These regions are known to be involved in REM sleep regulation [62]. In addition, the serotonergic projections from the raphe dorsalis [63], which are involved in NREM/REM sleep cycling [62], constitute a potential pathway for feedback of vigilance state onto the SCN. Moreover, the vigilance state modulates the SCN firing rate [64] and animal data reporting

a circadian phase difference [65] and different clock gene expression [66] after sleep deprivation indicate that sleep and wake states are able to influence circadian rhythms.

1.1.2.3 Clock genes and sleep

The genes that are responsible to drive circadian oscillations, clock genes, could also underlie the sleep–wake-driven relaxation oscillator that represents the sleep homeostat. These clock genes are transcriptional regulators and their nature makes them well suited not only to keep track of waking activity and sleep time but also to influence processes that may underlie sleep function at the cellular level. In addition, *Per1*, *Per2*, *Per3*, *Npas2*, *Clock* and *Bmal1* all encode proteins containing one or more so-called PAS signal-sensor protein domains. Based on both functional and phylogenetic considerations, PAS containing transcriptional regulators can be classified as either α-class proteins that act as sensors of environmental signals or as β-class proteins that act as more general dimerization partners [67]. The PER proteins, NPAS2 and CLOCK qualify as α-class PAS proteins whereas BMAL1 is considered a β-class PAS protein. Many PAS-domain proteins can sense oxygen, redox, voltage or light and are implicated in environmental and developmental signalling pathways.

To better understand the reciprical influence of clock genes and sleep, animal and human studies tried to unravel for several years the sleep phenotype caused by a genetic mutation. Bmal1$^{-/-}$ mice showed increases in total sleep time, sleep fragmentation and EEG delta power under baseline conditions and an attenuated compensatory response to sleep deprivation [68].

Mice lacking *Npas2* were found to sleep less in the latter half of the dark period, a time of day when sleep need is high conceivably to discharge accumulated sleep pressure [69]. When sleep pressure was increased by means of an 8 hours sleep deprivation, Npas2$^{-/-}$ mice were incapable of initiating the appropriate compensatory behavior (i.e. sleep) during the circadian phase in which mice are usually awake, i.e. the dark period [69].

Mice lacking the transcription factor *D-site albumin promoter-binding protein* (*Dbp*), a clock-controlled gene [70], showed decreased NREM sleep consolidation and EEG delta power and a reduced compensatory rebound in REM sleep after sleep deprivation [71].

From studies on clock gene mutant or knock-out animals, *Per* genes were found to have a major potential to modulate sleep. In knock-out animals *per1* and *per2* expression levels positively correlates with sleep homeostasis: the arrhythmic in dark/dark (DD) Cry1/2$^{-/-}$, whose *per1* and *2* levels are increased, shows an increased sleep need [66, 72-73],

whereas the opposite case is *Clock* knock-out animals, whose *per1* and *per2* levels are decreased, have less sleep pressure [70, 74]. On the other hand, sleep pressure is able to influence *Per1* and *Per2* expression levels: in sleep deprived animals, *per1* and *per2* levels are increased in the cortex, thalamus and cerebellum, but after only two hours of recovery sleep the Per levels drop down [66, 73]. However, only in the forebrain does *per* expression seem to consistently follow NREM sleep propensity, noticeably independent of the circadian oscillation of Per expression in the SCN [75]. In *Per1*,*2* double-mutant mice slow-wave activity seemed enhanced to a greater extent after sleep deprivation than in wild-type mice [76], suggesting that *Per* signaling is also functionally implicated in sleep homeostasis. In human studies *Per3* gene polymorphisms were shown to be involved in sleep homeostasis: individuals with $Per3^{4/4}$ polymorphism show a stronger decrement in performance sleep-loss-induced compared to $Per3^{5/5}$ individuals, suggesting a different susceptibility on sleep homeostasis [77].

1.1.3 Entrainment

In mammals, entrainment of the central clock in the SCN is an exclusively ocular process. Environmental light is transduced from the retina via both conventional rod and cone photoreceptors containing the photopigment rhodopsin and via a special class of retinal ganglion cells containing the pigment melanopsin [78]. These cells project directly to the suprachiasmatic nucleus. Without these retinal cells – e.g. in some totally blind or enucleated individuals – the circadian clock “free-runs” at a period length that often differs slightly from the solar day, leaving affected people with chronic jetlag-like symptoms [79].

Entrainmnent is defined as the alignment of a circadian system's period and phase to the period and phase of an external rhythm. Entrainment is the most common and a very important state for circadian system. In laboratory conditions single cues are studies, but the interplay between the intensities of different signals that occur in the wild is still far from being understood.

Signals that can entrain circadian system, also called zeitgeber (time cue), are: light, temperature, nutrients and non photic cues such as social entrainment.

In humans light is the most potent zeitgeber for the biological clock. Light pulses are able to reset circadian rhythm in a new phase. Moreover, the circadian rhythms of the majority of subjects that lack the physiological light responses (such as melatonin suppression by light) run free [80]. In other subjects lacking light responses whose circadian rhythms

period length is close to 24 hours, other weaker zeitgebers, such as social time cues, can entrain circadian rhythms [81].

Temperature, that can contribute to the human sleep-wake cycle rhythms [82] and nutrients are other zeitgebers whose abilities to entrain circadian rhythms are higher in lower living being such as *Neurospora crassa* [83] or *Gonyaulax polyedra* [84].

Masking is another parameter very important in real life. This is the case of signals that affect the overt circadian rhythms in a positive (enhancing) or a negative (depressing) manner but they are not related to the process of entrainment. An example of masking effect is periodic light-dark rhythms with period length between 23 and 25 hours, which constrain the sleep-wake cycle rhythms in a period length that is not physiological. Indeed, when subjects are kept in constant dark condition, their sleep-wake behaviour free-run, revealing the masking effects of light [85].

1.1.4 The chronotype

People vary in their diurnal preference for the timing of activity and sleep and this preference is paralleled in their physiology. The human phase of entrainment is called the "chronotype" (Fig. 2) and is largely regulated by the circadian clock. Several questionnaires were developed to assess the human chronotype. The most used questionnaires are the Horne-Östberg rating questionnaire [86] and the Munich Chronotype Questionnaire [87].These questionnaires score the subjects in morning, normal or late types on the basis of information about sleep-wake behaviour and propensities during working or free days. In morning types, wake time, the temperature nadir, and the plasma melatonin rhythm occur at earlier clock times than in evening types [88-89] (see chapter 1.4 for further details about melatonin). These aspects are consistent also when the subjects are in conditions without time cues and in absence of sleep.

Chronotype correlates with circadian period length: circadian period of subjects with different chronotype was correlated with chronotype such as that early types show a shorter period length than evening types [90]. The same finding was found analysing the period length of fibroblast circadian rhythms [91]. Moreover, fibroblasts harvested from early and late types with the same period length show significant changes in other circadian parameters such as amplitude and phase shifting properties, suggesting that human chronotype may be influenced also by amplitude and phase of circadian components, as well as by the period length [91].

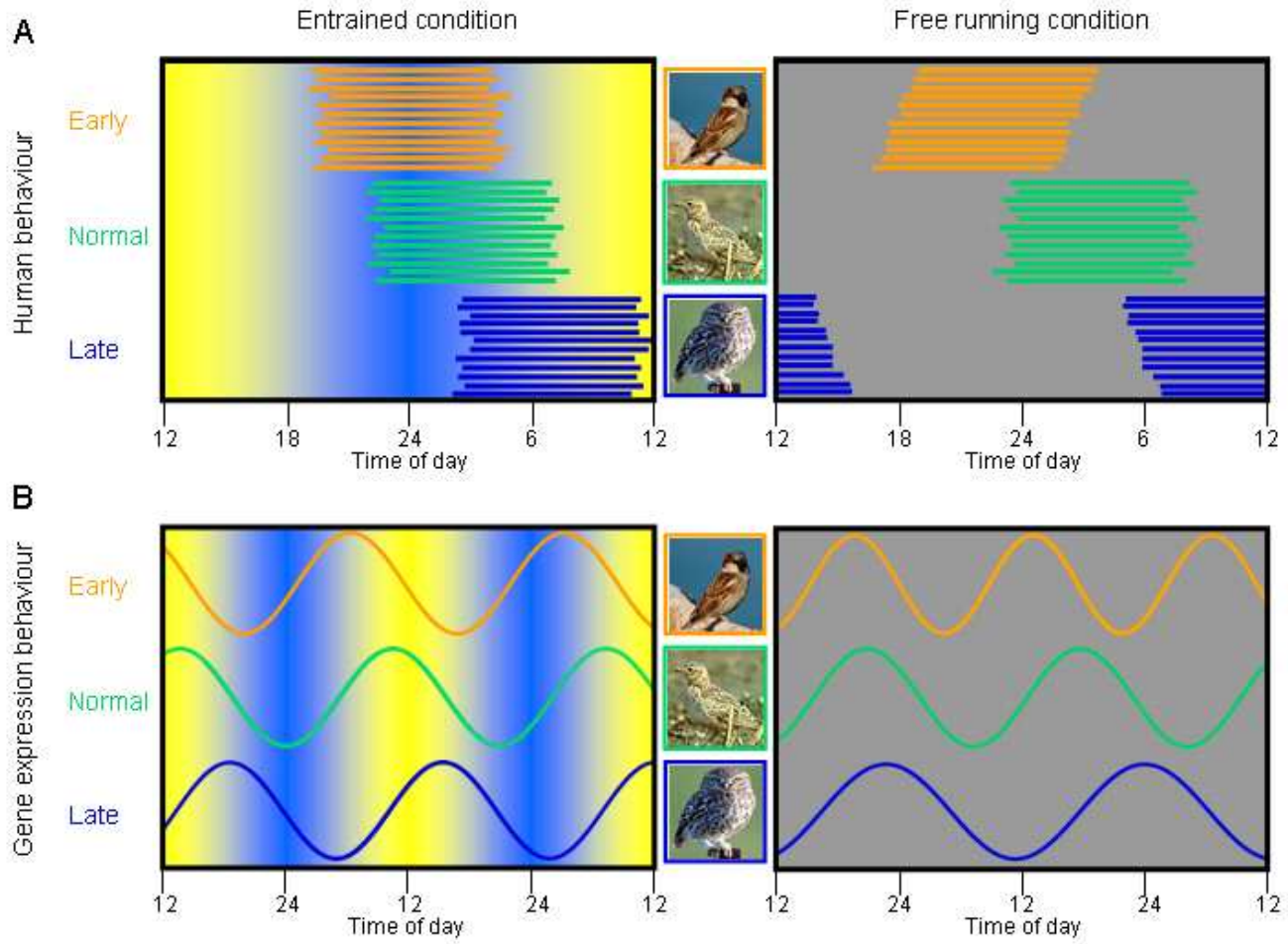

Fig. 2. Chronotype. Upper panel: schematic representation of 12 days of human sleep-wake cycle in three different types of chronotypes (early types in orange, normal types in green, late types in blue); bars represent hypothetical 8 hours of sleep. Lower panel: gene expression behaviour from the three different chronotypes. **(A)** In entrained condition (left), such as in real life, early chronotype (like larks), have an earlier phase of sleep compared to normal chronotypes, or late chronotypes (like owls). Without time cues (right), in free running condition, the period of the three "birds" is different: larks show a period shorter than 24 hours, normal chronotypes have a period of about 24 hours, while owls possess a period longer than 24 hours. **(B)** As well as human behaviour, gene expression in the different chronotypes has the same trend: in entrained conditions (left) the early chronotypes have an earlier phase of expression compared to normal or late chronotypes, while in free running conditions (right) larks have a shorter period than normal chronotypes or late chronotypes. (Adapted from [92]).

1.1.5 Circadian rhythm sleep disorders

Since the human sleep-wake cycle is generated through interactions of circadian rhythmicity, sleep homeostasis, entrainment properties, as well as feedback from the sleep-wake cycle onto these processes, the alterations in these processes and their interactions may lead to sleep and wakefulness occurring at abnormal clock times (altered external timing) and/or out of phase with endogenous circadian rhythms (altered internal timing).

The so-called "circadian rhythm sleep disorders" can result from alterations in the properties of the endogenous circadian clock (e.g., delayed sleep phase and advanced sleep phase) or changes in the physical environment in relation to the endogenous clock (shift work disorders and jet lag). When internal (circadian clock) and external time are not aligned circadian pathologies can occur.

1.1.5.1 Delayed sleep phase disorder

Althought their sleep architecture is normal, subjects affected by delayed sleep phase disorders (DSPD) have a the sleep schedule, nadir of the core body temperature (CBT) rhythm and dim light melatonin onset delayed by 3 to 6 hours compared to a normal sleep schedule [93-98]. The chronotype of these subjects is “evening” types [99-100]. The prevalence of this pathology is around 0.17% in the population [98], with a higher incidence in adolescents and young adults [98], even if it increases till 5-10% considering chronic insomnia patients [93].

There are some evidences that the etiology of DSPD has genetic causes: some polymorphisms in clock genes have been shown to be more or less associated to DSPS, such as *Per3* polymorphism containing 4- or 5-repeat sequences of potential CK1ε phosphorylation, or *Clock* polymorphisms [101-104]. Other causes of this pathology may be an unusually long endogenous circadian period [105-106] or alteration in the entrainment mechanisms of the circadian clock to synchronizing agents such as light that may lead to a higher suppression of nocturnal melatonin [107]. Other possible explanation for the causes of DSPD are an advanced portion of the phase response curve (PRC) to light abnormally small [108] or a lack of exposure to light in the phase advance region (due to sleeping later in the morning) [97].

1.1.5.2 Advanced sleep phase disorder

On the contrary of the DSPD, subjects affected by advanced sleep phase disorders (ASPD) present a sleep schedule and circadian parameters advanced by several hours compared to a normal subject. The chronotype of these subjects is “morning” types. The analyses of the polysomniogram shows decreased sleep latency, decreased total sleep time, and moderately short rapid eye movement (REM) sleep latency [109].

ASPD is often associated with aging [110] and may be caused by changes in the ability to phase delay as a result of a dominant phase advance region of the PRC to light or decreased exposure or weakened response to entraining agents, such as light and physical activity [111]. The prevalence of non-age-related ASPD is believed to be rare

[112]. In the reported cases of familial ASPD the disease segregates with an autosomal dominant mode of inheritance [113] indicating a genetic influence on the etiology of the pathology. In a family the source of the circadian change has been mapped to a change from serine to glycine at residue 662 of the *Per2* gene. This mutation leads to a reduction kinase activity of CK1ε [114], resulting in a shortening of period length of circadian rhythm [115]. In another family, a mutation in CKIδ gene was reported [116].

1.1.5.3 Free-running disorder

Free-running disorders occur when the entrainment with the external environment is not effective: this is the case of most of blind individual lacking the photopigments contained in rods, cones and retina ganglion cells [117], whereas in sighted individuals this pathology is less common and has in some cases arisen following head injury [118], or after a scorrect chronotherapy to treat DSPD [94, 119]. Free-running disorders are then characterized by periods of good sleep (i.e., long duration sleep, no daytime napping) during the days in which the non-entrained circadian pacemaker is in phase with conventional sleep and wake times and periods of poor sleep (i.e., short duration sleep with daytime naps) that occur when the internal circadiadian rhythms is misaligned compared to the external environment. In subjects affected by free-running disorders napping has been shown to be good indicator of a desynchronised circadian system: napping increases during an abnormal circadian phase and decreases during a normal circadian phase. Measurement of circadian rhythm markers, such as dim light melatonin onset or the CBT minimum at regular intervals may be used to confirm the diagnosis by demonstrating a drift in circadian rhythms other than sleep [120].

Other potential causes of free-running disorder include decreased sensitivity of the circadian clock to light, or an alteration in entrainment pathways that result in weakened or lack of entrainment of endogenous circadian rhythm [121].

1.1.5.4 Irregular sleep-wake rhythm

Irregular sleep-wake rhythm (ISWR) is a rare circadian disease characterized by a very poorly consolidation of sleep that leads to several sleep episodes every night [122]. Causes of this pathology may be a poor sleep hygiene, a voluntary irregular sleep schedule, a reduced exposure to external zeitgebers, such as light and activity, that is often seen in institutionalized care, or when irregular sleep-wake rhythms is a comorbidity to other major pathologies, such as dementia, mental retardation in children and brain injury [123-124].

1.1.5.5 Shift work sleep disorder

Shift work is another phenomenon that causes misalignment between internal and external time. The impact of shift work on sleep and daily functioning varies and not all shift workers have difficulties [125]. There are several factors, both internal and external, that can influence the ability to cope with shift work including age [126], domestic responsibilities [127], commute times, diurnal preference, type of work schedule, and other sleep disorders (e.g., sleep apnea, narcolepsy). Working on atypical shifts has important socioeconomic effects, since an estimated 20% of the workforce in many industrialized countries is employed in a job that requires shift work and it leads to an increased risk of accidents [128-131]. Shift work has been associated with a number of health problems including peptic ulcer disease, coronary heart disease, metabolic syndrome, certain cancers, undesirable pregnancy outcomes as well as increasing or aggravating of an existing medical condition [132-133]. Night shift work is also associated with an increased perception of mental and physical fatigue [134].

1.1.5.6 Jet lag

One of the most obvious manifestations of the misadjustment of circadian phase is due to travel through time zones. Flight dysrhythmia, more commonly known as jet lag, comprises a constellation of symptoms consisting of daytime fatigue, impaired alertness, nighttime insomnia, loss of appetite, depressed mood, poor psychomotor coordination and reduced cognitive skills [135]. The severity of jet lag symptoms is affected by both the number of time zones crossed and the direction of travel. Eastward travel tends to cause difficulty in falling asleep while westward travel usually interferes with sleep maintenance [136]. Not all travelers crossing time zones suffer from jet lag to the same degree. These differences probably result from individual variation, similar to those reported for adaptation to shift work.

The severity of jet lag symptoms has also been found to be greater in older individuals, inasmuch as proneness to illness and physical stress generally tend to increase with age [137-138]. A subtype of the jet lag is the “social jet lag”. Social jet lag occurs when social schedule modifies considerably individual sleep preferences [139]. The disruptive effect of jet lag has been documented at the molecular level of clock genes in the SCN [140] as well as in clock genes present in peripheral tissues [141]. Experiments indicate that endogenous circadian rhythms are lost and grafted tumors develop more rapidly in mice subjected to such chronic jet lag schedules [142].

1.1.5.7 Seasonal affective disorder (SAD)

SAD (winter depression), is a syndrome characterized by recurrent depressions that occur at the same period every year [143]. Depressive phases are associated with hypersomnia, overeating, and carbohydrate craving. Abnormalities in circadian rhythms in SAD include sleep disturbances [143] and alteration of many circadian rhythm markers secretion [144-148].

1.1.5.8 Circadian disorders in depression

One striking feature of circadian rhythm sleep disorders is that they are often associated with other mood disorders. Indeed, a part of this association is by definition: an established clinical symptom of diseases like major depressive disorder (MDD) and bipolar disorder (BD) is abnormal sleep/wake, appetite, and social rhythms [149-150] which are also hallmarks of circadian rhythm disorders. Nevertheless, an increasing body of evidence suggests that there exists an interesting genetic basis for this correlation. Several circadian markers show abnormalities in depression [151-155]. One hypothesis of the involvement of circadian rhythm disturbances and development of depression is that, perhaps, depression may involve a weaker coupling process between internal pacemakers and involve abnormal sensitivity to environmental cues such as light [153]. This could be a result of mutant clock genes or allelic variations, leading to abnormal clock cycles or altered photosensitivity. In BD, a single nucleotide polymorphism in the 3' flanking region of the Clock gene associates with a higher recurrence rate of bipolar episodes [156]. This mutation is specific to bipolar depression: a similar association is not found in MDD (or unipolar depression) [157]. Another mutation, this time linked to the onset of illness in BD, has been localized to the glycogen synthase kinase 3β promoter [158]. This enzyme is the target of lithium, a common treatment for BD, and can phosphorylate the clock component REV ERBα [159].

1.1.5.9 Treatments

Prolonged disruption of circadian rhythms, or misalignment compared to the external environment, is believed to have significant adverse health consequences (i. e. development or exacerbation of cardiovascular disease and cancer [160-162]). Chronotherapy and other forms of manipulation of the zeitgeber or sleep were demonstrated to be very effective for the treatment of circadian disorders pathology, or circadian disorders associated pathologies.

Chronotherapy is the progressive manipulation of sleep times to adjust it to a final normal bedtime schedule [93]. Another effective acute treatment for depressed and SAD patients is advancing the sleep and awake times on two occasions by 6 hours earlier than normal for a period of two weeks [163]. Sleep deprivation is also another effective treatment for unresponsive to antidepressant drugs depressed subjects. After one night of sleep deprivation many depressive symptomps are remitted [164].

Bright light therapy is another effective treatment that involves the use of bright light in specific time window to reset the human circadian clock and increase the entrainment of the subjects [165]. Exposure to bright light in the morning for 1 to 2 hours results in an advance of the phase of circadian rhythms, whereas evening light exposure causes phase delay. When SAD and depressed patients are exposed in the morning to bright light this elevates mood and stabilises circadian rhythm markers [147]. Although timed bright light appears to have potential utility, the timing, intensity and duration of treatment remain to be defined.

To adjust the sleep-wake timing to a normal schedule it is possible to perform pharmacotherapy with hypnotics or with melatonin. Administration of the hormone melatonin at specific times of the day can also reset the circadian clock in humans [166]. Several studies have demonstrated the potential effectiveness of melatonin administered especially to DSPD [167-168] and free-running individuals [169] (for further details about melatonin functions see chapter 1.4).

A strategy to block relapse in many patients includes therapeutic interventions combining sleep deprivation, sleep-wake cycle phase advance and light therapy.

1.2 ORGANIZATION OF THE CIRCADIAN SYSTEM

In complex organisms, like mammals, the circadian system is organised in a hierarchical manner: the master clock receives light information that elaborates and sends output signals to synchronize all the other peripheral oscillators.

1.2.1 The master clock

The SCN of the anterior hypothalamus (Fig. 3) is the site of a master circadian clock in mammalian brain [170] (see also 6.1 Appendix 1). Indeed, SCN lesioned animals are arrhythmic in entrained condition [171]. A seminal demonstration of the clock function of the SCN was made possible using the *tau* mutant hamster [172] (for further details see paragraph 1.2.3.1). The *tau* mutation shortens circadian period from about 24 hours in the

wild type to about 20 hours in homozygote mutant animals. Results from *in vitro* experiments with SCN slices have also shown that the *tau* mutation is expressed at the level of the circadian clock in the SCN [173]. *Tau* mutant hamsters thus were used in SCN transplantation studies to show that the period of the restored rhythm is determined by the genotype of the donor, not the host [174].

The record of spontanueos action potential from individual SCN neurons gave the first demonstration that the oscillatory machinery of the SCN is cell autonomous [175]. Indeed SCN dispersed neurons in the same culture express circadian rhythms of widely different phases and different period lengths despite functional synapses. The mean of the circadian period length was very similar to the mean of the period length of behavioral rhythms (studied in constant darkness) in the same strain of rat [175-176]. However, the standard deviation was higher in SCN cells compared to animal behaviour. A possible explanation of this difference is that the free-running period *in vivo* is determined by the mean period of clock cells in the SCN. The interactions among clock cells in the whole SCN serve to coordinate or synchronize individual circadian clocks to generate a common circadian output. A proposed oscillator network model shows that a completely synchronized network will oscillate at a frequency close to the sample mean of the frequencies of the population [177]. The oscillators near the centre of the distribution lock together in frequency, whereas the outlying oscillators continue to oscillate near their natural frequency. With further increases in the coupling, more of these outlying oscillators are pulled in, until eventually the whole system is synchronized. A hypothesis for the lacking of coupling in isolated cells is the eventual dilution of some diffusible substances necessary for coupling that occur in culture system. A biological advantage of a coupled multioscillator system is the flexibility that is provided in terms of entrainment of the circadian clock. This oscillator network model predicts that when a portion of the oscillators in the network are phase-shifted, the whole network will resynchronize to a new phase. Therefore, only a subpopulation of clock cells needs to respond to a specific entraining stimulus to generate a phase-shift in the whole network.

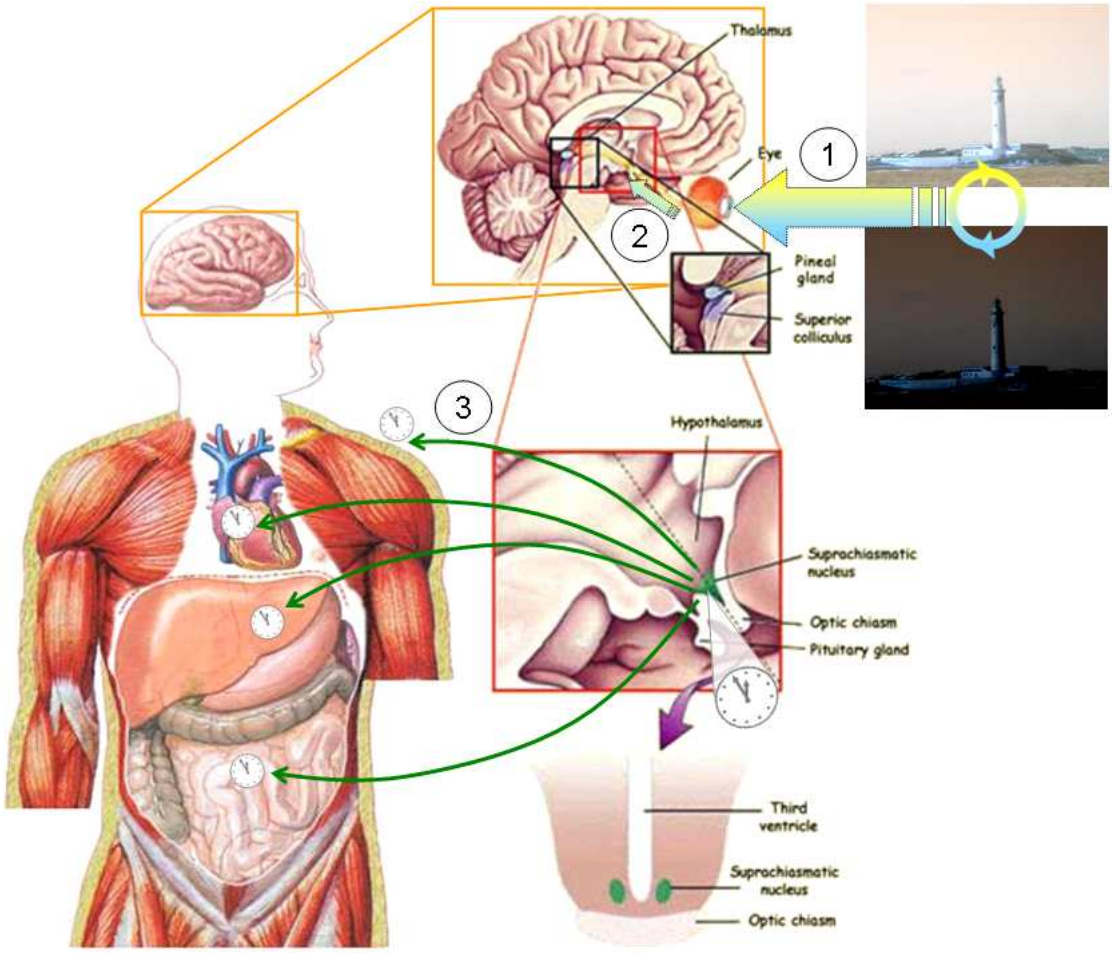

Fig. 3. Circadian rhythm hierarchy in mammals and humans. Environmental light is detected by the retina **(1)** that, through the retina-hypothalamic tract **(2)**, sends signals to the master clock, the suprachiasmatic nucleus (SCN) of the hypothalamus. SCN elaborates these light information and synchronizes its circadian rhythm with the external light-dark rhythm. Moreover, through different stimuli **(3)**, SCN synchronizes all the peripheral oscillators, virtually all cells of the body.

The cellular heterogeneity of clock properties probably serves a definite function. Although the day length on our planet is fixed to 24 hours, the period of light during this time can vary dramatically in lateral clines. To cope with these differences, the circadian clock is able to track both light onset (dawn) and light offset (dusk). Because of cellular differences in the SCN, some of its cellular clocks are phased earlier, thereby “track” dawn, while others are phased later to track dusk – a hypothesis formed long before the existence of cellular clocks was discovered [178] and recently confirmed in rodent SCN neural activity studies [179] and luciferase imaging experiments [180]. To keep SCN cells locally synchronized, neurochemical coupling mechanisms are used [181-182] and probably gap junctions as well [183]. Elimination of these components in mice results in dramatically dampened circadian oscillations.

1.2.1.1 Coupling mechanisms

Although the precise nature of the coupling interaction among SCN neurons is not yet known, one reasonable neurochemical candidate for such coupling activity is γ-aminobutyric acid (GABA). It is known that most SCN neurons are interneurons and they interact with each other within the SCN. Virtually all SCN neurons are GABAergic and respond to GABA [184-186]. The recent discovery that GABA differentially affects SCN neuronal firing, depending on the time of day of application, supports the synchronizing potential of GABA [187]. Moreover, GABA analogs phase shift the circadian clock both *in vivo* and *in vitro* [188-189]. It is also possible that diffusible substances other than GABA, such as nitric oxide (NO) or even neuropeptides, or a combination of these factors, serve as coupling factors among SCN neurons. Glia, through an insulating and/or a biochemical interaction, may also affect coupling among clock cells [190].

The SCN responds to timing signals received from afferent pathways and drives overt circadian rhythms through its efferent connections [191-193]. The SCN consists of neurons that synthesize a variety of neuropeptides, including vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), somatostatin (SS), and these molecules appear to play multiple roles in circadian timekeeping [194-195]. AVP and SS have been shown to modulate the function of the SCN. AVP neurons provide endogenous excitatory tone to the SCN [196], whereas SS administration inhibits SCN neuronal firing *in vitro* [197]. SS neurons are also involved in regulating the phase of circadian rhythms. Depletion of SS induces phase advances in locomotor activity rhythms and SCN electrical activity rhythms and permits rhythmic release of VIP from the SCN *in vitro* [198-199]. The demonstration that VIP neurons receive synaptic inputs from the retinohypothalamic tract, the geniculohypothalamic tract and the serotonergic projection from the median raphe nucleus suggests that the VIP neurons are involved in entrainment of the circadian pacemaker [200-202]. This concept is supported by finding that VIP microinjections in the SCN region induce phase-dependent phase shifts in locomotor activity rhythms [203-205].

1.2.1.2 Anatomy of the SCN

The SCN can be divided into two regions, a core and a shell, which have different properties [206-207]. Cells of the core receive light information via the retina-hypotalamic tract (RHT) [208] whereas cells of the shell show intrinsic rhythmicity but are only little innervated from the retina via the RHT [209]. When the majority of core cells is ablated, circadian rhythmicity is lost. SCN core cells seem to gate photic input depending on their

internal phase, thus leading to differential phase shifts of rhythmic cells of the SCN shell [210].

1.2.1.3 Input signals of the SCN

The different responsiveness of the SCN is enabled by a daytime and a nighttime domain [211] at which only certain substrates efficiently adjust clock phase [211]. In the early night, sensitivity to light is mediated by glutamate and elevation of intracellular calcium levels [212], correlating with the timing of phase delays [213-214]. Responsiveness of the SCN to pituitary adenylate cyclase-activating peptide (PACAP) might modulate nocturnal responses both to light and to glutamate [215]. In the late night, glutamate induces phase advances by a cyclic guanosine monophosphate (cGMP) mediated pathway [213, 216]. During dawn and dusk, the SCN shows responsiveness to melatonin [217], a hormon produced during night mainly in the pineal gland [218]. (See in the next chapters for SCN responsiveness to melatonin).

1.2.1.4 Output signals of the SCN

The SCN “master clock” is entrained by light and it in turn entrains “slave” oscillators located in several brain areas and the periphery via a myriad of redundant cues. One important class are direct nervous signals. The neurons of the SCN demonstrate spontaneous firing patterns in daily fashion and project to many other brain nuclei, where they are presumed to be important for sleep-wake cycles and cognitive function. Many hypothalamic nuclei receive SCN projections and mediate the circadian control of multiple systemic cues. For example, both direct and indirect projections from the SCN make synaptic contact with corticotropin-releasing hormone (CRH) neurons of the paraventricular nucleus (PVN). These neurons in turn impose rhythmic adrenocorticotropic hormone (ACTH) release from the pituitary and subsequent circadian corticosterone secretion from the adrenal glands [219]. The sleep/arousal, reproductive, and endocrine systems are also regulated by the SCN in part through neuroanatomic connections. Hormonal signals are capable of entraining peripheral oscillators, as glucocorticoids agonists can effectively shift peripheral clock gene expression in mice [220]. The precise role of other cyclically secreted hormones such as growth hormone (GH) and prolactin (PRL) in peripheral phase entrainment is yet to be examined. In addition, it has been demonstrated the existence of SCN connections to all major organs via both sympathetic and parasympathetic pathways, which implies a critical role of the autonomous nervous system in synchronizing peripheral physiology [219]. The SCN reaches the autonomic

neurons of the PVN that project to the intermediolateral column of the spinal cord, where preganglionic sympathetic neurons are located [221].

Paradoxically, the first line of communication from the SCN to centers controlling locomotor activity is probably hormonal: animal experiments show that implantation of SCN neurons encapsulated in porous plastic are still capable of rescuing rhythmicity in SCN-lesioned animals [222].

A second class of signals is indirect products of the regulation of other brain centers by the SCN. Body temperature is one important class: even though daily body temperature varies by only 1-4℃ in mammals, these faint daily fluctua tions – probably controlled by SCN enervation of the preoptic anterior hypothalamus and by daily activity patterns – are sufficient to entrain peripheral tissues [223]. Similarly, daily patterns of feeding are likely cues for the entrainment of peripheral clocks in tissues throughout the body [224-225], as well as a separate “food-entrainable” brain oscillator that can control locomotor activity in the absence of the known circadian clock [226-227]. In animals, alterations of either of these classes of signals effectively “decouples” the central clock from oscillators in other tissues: either reversal of daily body temperature rhythms or of daily feeding rhythms can inverse the timing of local clocks in peripheral organs such as liver, kidney, heart, and pancreas, leaving SCN rhythms unaffected [224]. It was then hypothesized that, besides the light entrainable circadian clock, another clock exists which can be entrained by food, the food entrainable oscillator (FEO) [228]. Brain-regulated corticosterone rhythms probably serve as a stabilizing influence: in animals lacking the glucocorticoid receptor in the liver, hepatic clocks are much more rapidly shifted by alterations in daily feeding patterns [229]. Hormones and metabolites related to feeding, such as insulin and glucose, are likely involved in the phase setting of peripheral clocks [230].

Activity rhythms as well as circadian outputs from other tissues can feed back on the SCN and on other peripheral oscillators. For example, melatonin released from the pineal gland, which is itself under SCN control through autonomic pathways, functions as a circadian input signal for both the SCN and the anterior pituitary [231].

1.2.2 Peripheral oscillators

When the first core genetic components of the mammalian circadian clock was identified, it was discovered that these “clock genes” are expressed rhythmically outside the SCN [232] and even outside the brain [233] in many peripheral tissues [234] (see also 6.1 Appendix 1). The use of a transgenic *Drosophila* that expresses either luciferase or green

fluorescent protein driven from the promoter of the clock gene *period* permitted to follow in real-time the oscillation of circadian rhythm in peripheral tissues such as head, thorax, and abdominal tissues [235-237]. These rhythms are entrainable by light and free-run in constant darkness [235]. From dissociated cells of different body segments (head, thorax, or abdomen) rhythmic bioluminescence was obtained, demonstrating that autonomous circadian oscillators are present throughout the body of a fly.

Presence of circadian rhythm in several peripheral tissues has been widely demonstrated also in mammals. The mRNA expression of main clock genes (*mBmal1*, *mNpas2*, *mRev-erbα*, *mDbp*, *mRev-erbβ*, *mPer3*, *mPer1* and *mPer2*) have been examined in cells obtained from different peripheral systems (heart, lung, liver, stomach, spleen, kidney, and testis) [238]. Circadian mRNA expression patterns were similar in each tissue with the exception of testis, where weak or no rhythm was observed. In the peripheral tissues mRNA peaks occurred approximately 4 hours later than those in the central pacemaker, SCN [24, 233, 239-242].

Real-time clock gene expression of peripheral tissues can be obtained through explants of peripheral tissues from transgenic *mPer1::luc* rats [243] and in transfected fibroblasts [244-245]. Rhythmic clock gene expression can even be found also in immortalized mammalian cell lines like Rat-1 fibroblasts [246]. Unlike the SCN explants, the peripheral cells or tissue rhythms tend to damp out within a few cycles until restarted by serum shock or medium change.

The difference in damping between SCN and other tissues appears to be quantitative rather than qualitative. However through single cell resolution, detecting bioluminescence of Rat-1 fibroblasts acutely transfected with an *mBmal1::luc* plasmid and primary fibroblasts dissociated from *mPer2-Luciferase-SV40* (*mPer2::Luc-SV40*) knockin mice [247] permitted the observation that damping is explained by loss of synchrony among cells rather than damping of individual cell rhythms.

1.2.3 Circadian parameters

It is possible to determine the genetic linkage between human behavioural disorders and the circadian oscillator by measuring the properties of the human circadian clocks that determine behaviour. Parameters that can be measured are free-running period, or the length of one oscillation under constant environmental conditions and phase

response/entrainment, the ability of the clock to alter its phase in response to external stimuli and the amplitude of the rhythm, that is the force of the oscillations (Fig. 4).

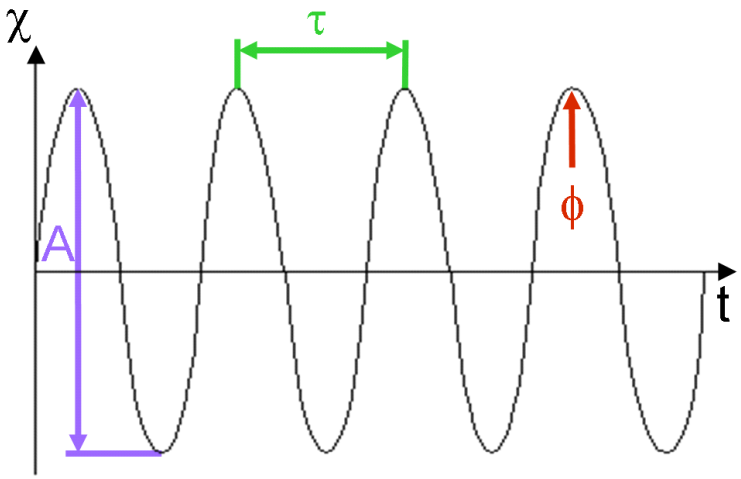

Fig. 4. Parameters of an hypothetical rhythm. Given a variation of an element "χ" in units of time "t", several parameters of a rhythm can be measured, such as amplitude (A, in violet, defined as the maximum absolute value of a periodically varying quantity), period length (τ, in green, the time spent between two peaks) and phase (ϕ, in red, that represents the position of a peak compared to the time unit).

1.2.3.1 Period length

One of the first connection that have been made between the behaviour of a living being and the behaviour of single cells comes from the analyses of *tau* mutant Syrian hamsters. Tau is a missense mutation within the substrate recognition site of CKIε (CKIεtau). CKIε and the closely related CKIδ are widely expressed serine-threonine kinases implicated in development, circadian rhythms and DNA metabolism. In CKIεtau the enzyme has a much reduced overall catalytic activity. The period length of the free-running behaviour is different from wild-type (+/+), heterozygous (*tau*/+), and homozygous (*tau/tau*) *tau* mutant Syrian hamsters. The mean periods of wild-type, heterozygous, and homozygous clock cells (electrical activity of single SCN neurons) correlated nicely with the respective circadian periods in behaviour (wheel running behaviour) in the whole animal [177].

Similarly, a strong correlation has been found between *in vivo* free-running behaviour from different mouse strains and period length of primary adult dermal fibroblasts of mouse tail. Mouse fibroblasts were infected with *Bmal1::luc* reporter gene. The period length of fibroblast was determined by the measurement of the bioluminescence emitted by the cells [248]. The advantage of this finding is that the central clock of the SCN that specifies behaviour is quite difficult to access at a molecular level whereas the sampling of peripheral cells, that appear to use many of the same components as the SCN, is more feasible also in human. Hence, a major breakthrough for mammalian circadian biologists

has been the ability to use peripheral oscillators as proxies—albeit imperfect ones—for the clocks of the SCN.

1.2.3.2 Phase

The phase of entrainment is the position of a parameter compare to the internal time. In human studies there are several parameters that can be chosen to determine the phase of entrainment. These parameters include temperature (the moment of minimum of the CBT), melatonin onset and melatonin offset. In cell lines the phase of entrainment can be determined by the analyses of the position of the peak of a circadian marker, such as *per* or *bmal-1*. It can also be determined in real-time using the luciferase reporter gene under a clock gene promoter. As for a circle the degrees of phase can be 360, with the convention that 0° is the phase of the fitted peak of the lumi nescence rhythm. Commercial fibroblast lines have the same phase behaviour respect to primary fibroblast. After synchronization of circadian rhythm the phases of individual fibroblasts are clustered. Losing of phase determines the damping of the circadian rhythm of peripheral oscillators. Indeed, after few days of bioluminescence recording the cells lose synchronization and the phase is randomly distributed [247].

1.2.3.3 Amplitude

Amplitude is the magnitude of change in an oscillating variable, with each oscillation, within an oscillating system. In human the amplitude can be defined for parameters such as melatonin and CBT. In cells parameters are gene expressions or protein oscillation.

1.2.3.4 PRC

Entraining signals advance or delay circadian rhythms according to the time they are experienced. The direction and magnitude of the change in timing (phase shift) is described by a PRC. The time base of a PRC is ‘circadian time’ (CT) which is effectively internal biological time usually defined in humans by the timing of the CBT rhythm or melatonin secretion. To entrain to the daily light/dark cycle, the circadian oscillator responds differently to light at different phases of its cycle. This differential effect is most easily visualized as a PRC, which plots phase shifts of a circadian rhythm as a function of the circadian phase that a stimulus is given. In biological oscillators, the "dead zone" (phase 12 and 24) is usually much larger and can span over several hours. PRCs are determined experimentally by first keeping the organisms of investigation (e.g. mouse) under constant condition, e.g. complete darkness (DD). Different organisms receive then a pulse of a stimulus with a certain duration and intensity at regular intervals within 24 hours.

The phase shift of the circadian marker, e. g. the onset of activity, is measured for every group of organisms and can be plotted as a PCR.

In human beings, the measurement of either the phase response is very expensive and labor-intensive because it demands extensive subject observation under controlled laboratory conditions. However the same phase response experiment can be conducted with cells. Cells are synchronized and after one cycle independent cell cultures are exposed to a phase-shifting agent at regular intervals within 24 hours. The plot of PRC is obtained analysing the position of the peak obtained treating cells at a certain time with the peak obtained without shifting-agent.

1.2.4 *In vivo* human studies

Several protocols were developed to reveal the endogenous circadian component of human rhythms. The major issue in the study of human circadian rhythms is the masking effect that are caused by light, temperature (environmental and CBT), body position, food intake, several hormones (i. e. cortisol) and many other factors. In the protocols developed to study human circadian rhythms all factors that may contribute to masking of circadian rhythms are controlled.

In these protocols one or more circadian markers are measured to investigate circadian rhythms. The analyses of the circadian rhythms of these markers can give hints about circadian parameters, such as amplitude, phase and period length. Melatonin, cortisol and CBT are the best circadian markers that used to investigate human circadian rhythms.

1.2.4.1 Circadian markers

CBT (Fig. 5) is a parameter that is very easy to measure and for this reason is one of the frequentely used to analyse human circadian rhythms. Its measurements can be done in continuous without much disturbances for the subjects through a rectal probe and data can be analysed in real-time. However, many factors contribute to mask the CBT. These factors include: behaviors such as postural changes, physical activity and meals, and also external conditions, such as ambient temperature, sound, humidity, and bright light [249-250]. Age is another factor that influences CBT: CBT nadir is earlier in elderly people compared to young people [251].

Cortisol: among the hormones that show a strong circadian rhythms melatonin and plasma cortisol are most frequentely used to investigate circadian rhythms. Within the cortisol rhythms the nadir (acrophase), onset of the evening rise and the start or end of quiescent

period are the mainly analysed cortisol parameters [252-253]. However, many factors are known to mask cortisol rhythms such as: physical and psychological stress, light, ageing, sleep onset and deep sleep and highly proteic meals [250].

Melatonin (Fig. 5) is another hormone whose cycles can be used to study human circadian rhythms. Melatonin levels can be measured in several biological fluids: in blood and saliva melatonin levels are proportional. Alternatively, the melatonin metabolite 6-sulfatoxymelatonin can be easily detected in the urine of subjects [254], which strongly correlates with the plasma and serum melatonin levels [255]. The dim-light melatonin onset (DLMO) is considered to be one of the most reliable markers, as it is thought to be minimally masked by exogenous factors. Melatonin can be masked by posture, exercise, sleep and sleep deprivation, caffeine, and certain drugs, for instance, nonsteroidal anti-inflammatory drugs (NSAIDs) and beta blockers [256-258]. However, these factors are easy to control in human studies (for further details about melatonin, see chapter 1.4).

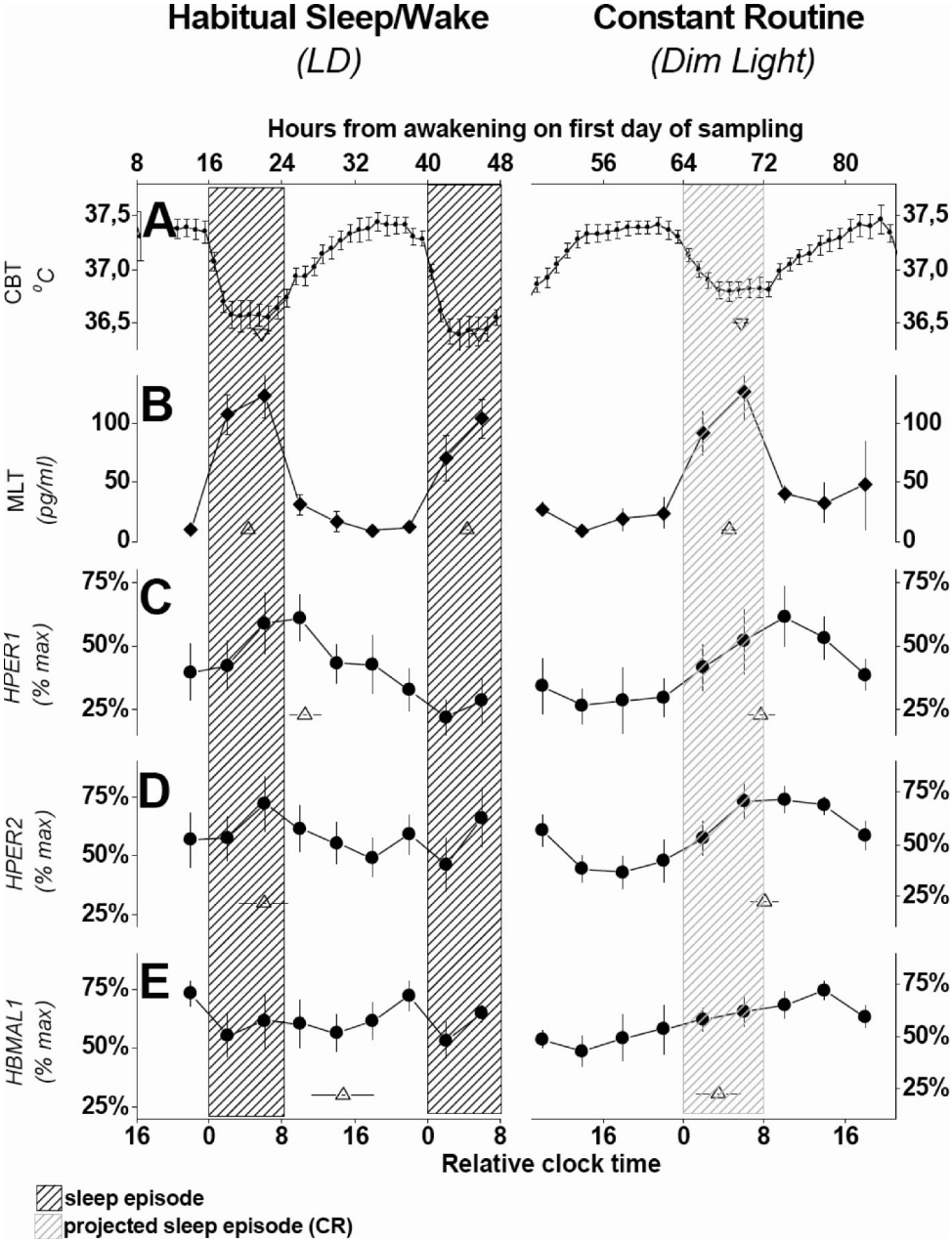

Fig. 5. Circadian expression of circadian markers in entrained (on the left, habitual sleep/wake cycle) and free running condition (on the right, constant routine) of 6 subjects. Triangles represent the mean of circadian phase for each marker. Grey bars represent the sleeping episodes; light grey bars represent habitual sleep periods. Bars represent SEM. (A) Core body temperature (CBT). (B) Plasma melatonin (MLT). (C-E) Respectively, gene ex-pression levels of human Per1 (HPER1), human Per2 (HPER2) and human Bmal1 (HBMAL1) in peripheral blood mononuclear cells. (From [259]).

1.2.4.2 In vivo protocols

Constant darkness and free-running protocol: Many of the early data on the human circadian system were collected on subjects studied in isolated environments free of time cues. Under these conditions, the subject's circadian pacemaker was believed to oscillate at its intrinsic period of ~25 h. The condition of an ambient that lacks time cues and other environmental agents that may affect the period of the oscillation is called free-running. Until recently the free-run protocol has been the "gold standard" for assessing the intrinsic properties of the circadian system. It is now appreciated that light, even at the level of ordinary room lighting, can shift the circadian pacemaker and that the period estimated under free-run conditions is not the intrinsic period of the human circadian pacemaker

[260]. This is because the human circadian pacemaker, like those of lower animals, has a well-defined phase response curve (PRC) to light [165]. Under free-run conditions, a subject self-selects his/her light-dark cycle and, hence, the phase at which the light is self-administered. Because of the asymmetric structure of the human PRC, this self-selection results more frequently in light administered during phases that favor delays of the human pacemaker, rather than advances, and therefore in an observed period that is longer than the pacemaker's intrinsic period. Alternatively, circadian period can be measured under constant dim light for several days without time cues [261]. In this way the shifting effects of light are reduced. A circadian parameter is measured at specific days for 24 hours and the period length is measured by interpolating the peaks of the circadian parameter.

A further special case employs totally blind individuals, whose circadian pacemaker cannot be entrained by the environmental light-dark cycle and who can therefore be measured during daily life because it free runs [79].

Constant routine: the constant-routine protocol controls environmental conditions and a subject's level of activity to identify accurately the circadian component of an observed biological rhythm [262]. This protocol was developed as an alternative to longer free-run studies, as a mean of assessing the properties of an individual's circadian system in a shorter study interval. The protocol calls for subjects to remain continuously awake for 30-60 hours in a semirecumbent posture exposed to constant indoor light (~150 lux) and to consume their daily caloric intake in evenly divided snacks at approximate hourly intervals [263]. Physiological and behavioral circadian variables are recorded and the properties of the circadian pacemaker are studied by characterizing the phases and amplitudes of these marker rhythms such as core temperature, plasma melatonin levels, and plasma cortisol levels [165]. The pacemaker period cannot be determined, because, at best, only 1.5-2.5 cycles of the oscillation are observed on this protocol [264-265]. An advantage of this protocol is that it minimizes the effects of environmental conditions and level of activity on a subject's observed circadian rhythm.

One major issue in the use of this protocol is that during all this protocol sleep pressure increases. To minimize the masking effects of sleep homeostasis a modification to the costant routine protocol was developed. In this protocol, called “multiple nap protocol”, alternating sleep-wake cycles of 75:150 minutes duration are scheduled for a total duration of the protocol of more than 60 hours. In this protocol the waking time are performed in constant dim light levels, semi-recumbent posture in bed, food and liquid intake at regular intervals, no access to time cues, whereas during the scheduled sleep episodes a supine

posture is allowed and the lights were turned off (0 lux) [266]. Virtually with this protocol it is possible to unmask circadian rhythms from the homeostatic modulation: indeed sleepiness exhibits a prominent circadian regulation [267].

Forced desynchrony: under 'forced desynchrony' conditions the sleep-wake cycle is scheduled to day-lengths that are outside the range of entrainment for the circadian pacemaker (longer than 27 hours or shorter than 21 hours) [260]. During two-thirds of the light-dark cycle, the lights are maintained continuously switched on at a fixed intensity (i. e. 20 lux or lower), whereas during the remaining one-third of the cycle, the subject is maintained in total darkness.The behavior of the clock is monitored by recording marker rhythms such as CBT, plasma cortisol, and plasma melatonin levels [260]. Because the light levels during forced desynchrony are low and because the clock cannot synchronize to a light-dark cycle whose period is outside the range of entrainment, the circadian pacemaker oscillates at its intrinsic period.

1.2.5 Cell culture and ex-vivo studies

In vivo human studies are very expensive and labor intensive. Moreover the techniques to measure circadian markes are often invasive, decreasing the compliacy of subjects to these protocols. Taking advantage of the presence of the clock circadian machinery in virtually all cells of the body, it is possible to perform circadian studies in cell cultures. The use of cells isolated from an animal or human tissue permitted the development and the validation of ex-vivo protocols.

Many studies of circadian-regulated gene expression require the accurate and long-term monitoring measurement of transcriptional responses to intra- and extracellular signals. Classical methods needing the destruction of tissue to measure steady-state RNA or reporter enzyme levels *in vitro* have significant limitations for temporal studies owing to noise and reproducibility [268]. Analysis of clock gene transcription is considerably more versatile in a real-time reporter system established for clock genes. The first reporter system that has been developed in *Drosophila* involved the measurement of *per* mRNA levels for reporter genes such as *lacZ* [269-270]; however β-galactosidase activity does not cycle when the *per* promoter is fused directly to *lacZ* [269]. This is most likely due to the approximately 1 day half-life of the enzyme in the fly [271].

A second reporter gene assay developed to study circadian rhythms involves the enzymatic reaction of luciferase [235, 272-274]. Luciferase-mediated bioluminescence is proving to be a convenient and powerful reporter in organisms ranging from microbes to

mammals [268]. Firefly luciferase has been the most common choice for the analysis of promoter function in eukaryotic systems [275-276]. The firefly enzyme catalyzes the oxidative decarboxylation of beetle luciferin using oxigene and magnesium ± adenosine-5'-triphosphate (ATP) as substrates. A photon is released at 560 nm in 90% of catalytic cycles: this light emission can be quantified with high sensitivity, most commonly in a luminometer. In mammalian cell cultures, the half-life of luciferase is approximately 3 hours [277-278].

A significant application of luciferase reporter has come from its use as real-time marker to detect real-time emission of bioluminescence of circadian rhythms and was first used in experiments aiming at identifying novel rhythm mutants [273, 279].

Luminescence imaging is also well suited to analyse gene expression of a transgenic mouse *in vivo* through whole-animal imaging [280].

Single-cell bioluminescence imaging has also been achieved in mammalian systems and has revealed important new insights about the cellular construction of the master circadian clock in the brain. Through the single-cell resolution and long-term monitoring of bioluminescence rhythms of SCN brain slices cultured from an *mPer1::luc* transgenic mouse it was possible to observe a stable pattern of phase differences among cells: in the SCN dorsal cells tended to peak earlier than ventral cells. When dorsomedial or ventrolateral portions of the SCN were isolated from each other, cells in both portions remained rhythmic, but dorsomedial cells drifted out of synchrony while ventrolateral cells remained synchronized. This observation suggests a role for the ventrolateral SCN in maintaining coupling of cellular oscillators throughout the SCN [281]. Single-cell resolution of bioluminescence can be obtained also from peripheral mammalian oscillators. Circadian rhythm can be monitored through bioluminescence rhythms for 1–2 weeks in Rat-1 cells transfected with an *mBmal1::luc* reporter and in primary fibroblasts cultured from *mPer2::Luc* knockin mice. After few days the circadian rhythm damping is observed in peripheral oscillators. Single-cell analyses of bioluminescence allowed finding that the damping of the ensemble rhythm observed at the level of a cell population resulted from a gradual loss of synchrony among independent cellular oscillators and not from the damping of individual cellular rhythms. Thus, individual fibroblasts contain self-sustained circadian oscillators, like SCN neurons [247].

1.2.6 Use of primary cells to study individual traits

After the evidence of the presence of a canonical circadian oscillator in established tissue culture lines it has been noticed that these oscillators may not be totally unrelated to the mammalian central oscillator but instead they possess several characteristic in common to the master clock cells (Fig. 6). Peripheral cells are able to maintain a robust oscillations that *in vivo* occurs with 4 hr phase difference between cycling in the SCN and cycling in the periphery [240]. Cycling mRNA undergoes an oscillation in the fibroblasts comparable to its *in vivo* circadian cycle after treatment with a synchronizing agent (serum shock). There are evidences of a connection between light-induction in the SCN of a living being and serum-induction in culture. A phase-shifting light stimulus is known to stimulate the transcription of a set of genes so-called immediate-early genes (IEGs) within the SCN. The same genes that are rapidly induced by light in the SCN are also rapidly induced after the serum shock in tissue culture. Both SCN and peripheral oscillators have the ability to free-run in the absence of environmental clues. And even if some damping turns out to be an intrinsic feature only of the tissue culture oscillators, the similarities between the two systems will almost certainly dwarf the differences. The ability to transfect peripheral oscillators such as fibroblasts with overexpression or dominant-negative constructs is another important tool that can be used to investigate particular aspects of circadian rhythm. In this way it has been possible to ensure that circadian genetic differences are reflected in the rhythms of fibroblast gene expression. Circadian rhythm of adult dermal fibroblasts was analysed from tail biopsies of mice with a different genome. Parallel to this analysis, circadian wheel running behaviour was measured for the same individuals. Mice with a period of wheel-running behavior shorter than wild-type yielded fibroblasts whose period of circadian Bmal1 expression was also shorter. Similarly, fibroblasts from mice with a period of wheel running that was longer than wild-type had correspondingly longer period lengths. Mice that were behaviorally arrhythmic produced arrhythmic fibroblasts [248].

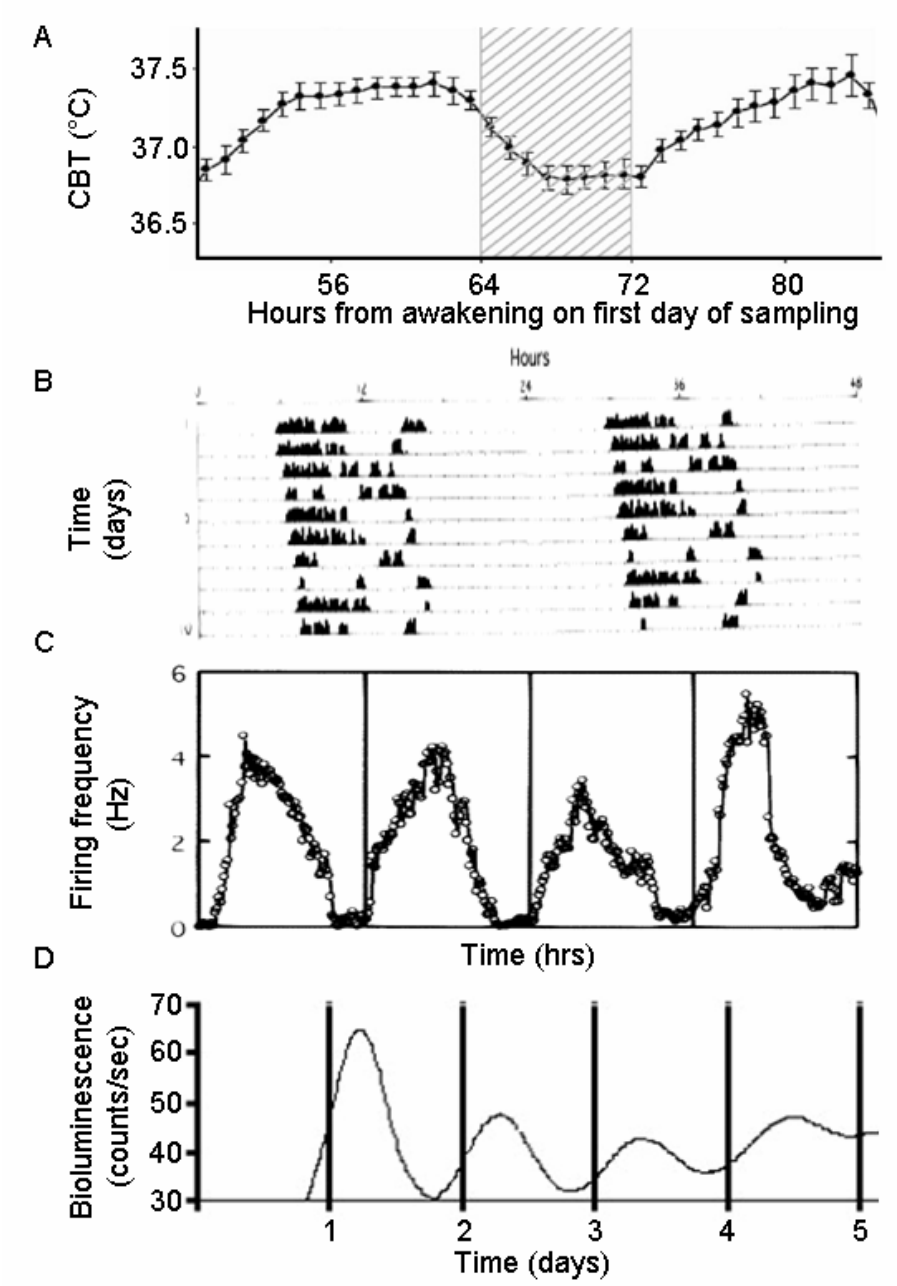

Fig. 6. Circadian rhythm in different models. (A) Core body temperature in humans; grey bars represent habitual sleep period. **(B)** Locomotor activity in hamster. **(C)** Hamster suprachiasmatic nucleus neuronal firing rate. **(D)** Human skin fibroblasts rhythm from *Bmal1::luc* infected cells. (Adapted from [259], [177], [91]).

Primary cells are a good model to study circadian aspects for many reasons. Some circadian characteristics, such as the period length (Fig. 7), remain unmodified even isolating *in vitro* the oscillators. However the measurement of the period length of the circadian rhythm of isolated cells or tissues is exaggerated compared to the period length of the all animal or the period length measured in SCN cells. One hypothesis of this phenomenon is the absence of coupling between peripheral oscillators [248, 282].

A big advantage in the use of peripheral oscillators is the possibility to manipulate the genome. It is possible in fact re-create the genetic mutation of a disease to investigate the molecular mechanisms that determine certain behaviour. Vanselow and colleagues

introduced a mutation in *Per2,* believed to be responsible for human familiar ASPD, into fibroblasts and were able to recapitulate the phase advance in the behavior of familiar ASPD patients as an advanced phase of clock-gene transcription in synchronized familiar ASPD fibroblasts. Subsequent molecular analyses allowed them to show effects of this mutation upon phosphorylation at multiple sites in the PER2 protein and to further demonstrate that these modifications affected both PER2 protein stability and nuclear localization [283].

In vitro studies on isolated peripheral oscillators can be associated to *in vivo* studies to investigate a circadian question from more points of view. A prerequisite for peripheral oscillators to be a good model to study circadian rhythm is to be easy to sample. Blood cells and skin fibroblast have this characteristic.

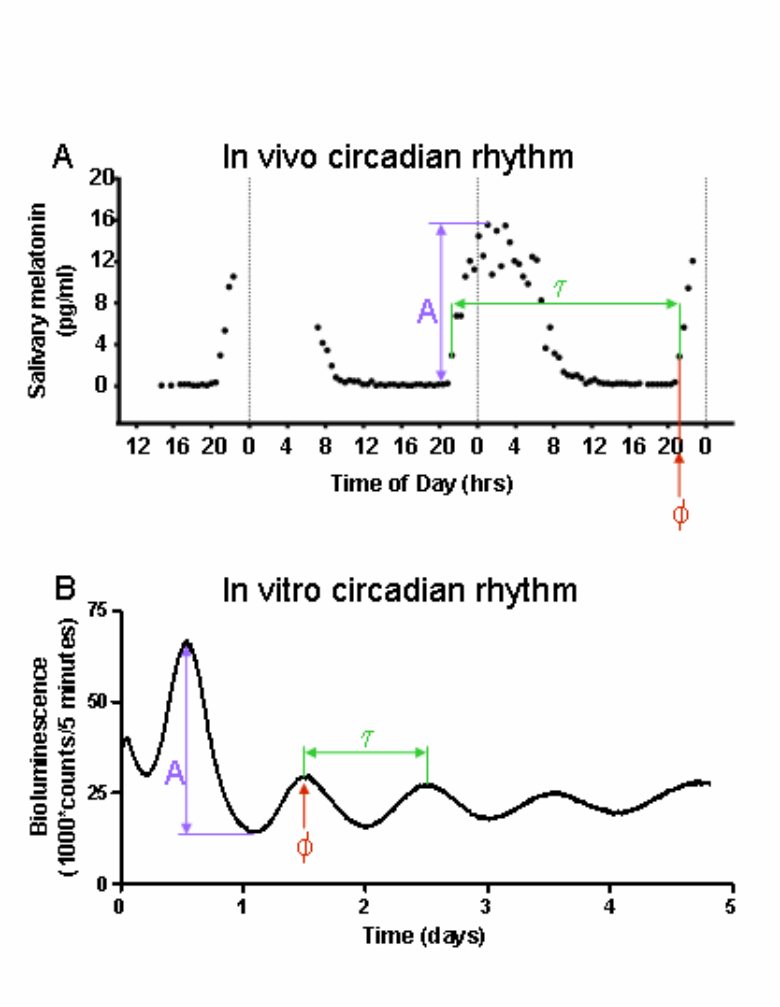

Fig. 7. Circadian rhythms analysed using a human *in vivo (A)* and *in vitro (B)* protocol. (A) Salivary melatonin content measured every 20 minutes in a constant routine. The following rhythmic parameters can be analysed: amplitude (A), period length (τ) and phase (ϕ). **(B)** Similarly, the same parameters can be analysed from the light emitted by synchronized *Bmal1::luc* human skin fibroblasts.

1.2.6.1 Blood cells

The hematopoietic and the immune systems in all their components are characterized by highly reproducible circadian rhythms in cell proliferation and cell function (circadian rhythmicity of cytokine production, leukocyte trafficking, proliferation, and apoptosis). Manipulating environmental parameters such as activity-rest, light-dark schedule and/or the time of food uptake can cause phase alterations in the circadian rhythms of hematologic cells. Characteristically these functions do not change their timing immediately after a shift in a time cue phase but adapt over several transient cycles [284]. The fact that nervous system has been shown to modulate the neuroendocrine system strongly suggests that circadian mechanisms modulate the immune response through signals ultimately regulated at the hypothalamic level.

The existence of the molecular machinery of circadian rhythm in blood cells has been widely demonstrated. Presence of both negative (*Per1* and *Per2*) and positive (*Bmal1* and *Clock*) components of the molecular clock have been observed in natural killer (NK) cells in both light-entrained rats [285] and in rats maintained in constant darkness [286]. The characteristics of clock transcript oscillations in NK cells are similar to those seen in other peripheral tissues.

Several evidences reveal that the expression of *Per2* in peripheral blood mononuclear cells (PBMCs) is significantly different in the morning and the evening, with an expression peak around dark phase (zeitgeber time (ZT)14) in rat [287] and around 9:00 a.m. in humans [288]. Interestingly, mRNA levels of *hclock* do not exhibit daily variation [288].

Further constant routine studies revealed the presence of oscillation of clock genes in PBMCs in the absence of external cues [289]. The analyses of circadian gene expression in mononuclear (MNC) polymorphonuclear (PMN) cells showed the same acrophase of *hper1* mRNA expression rhythm. PMNs exhibited an additional smaller peak in *hper1* expression in the subjective evening similar to that reported in human oral mucosa and skin cells [290], whereas this second peak was not observed in MNCs [291].

One advantage in blood cells as circadian tool comes from their versatile use, the feasibility of studying PBMCs as an accessible surrogate for the identification of rhythmic clock gene expression in humans and the possibility of performing genome modification. Activated human monocytes can be cultivated and infected with *Bmal1::luc* reporter gene, in order to detect real-time clock gene expression [248].

The demonstration of functional circadian machinery in human blood cells and the possibility to perform a knock-in cell line suggests that peripheral blood cells may be useful for the investigation of human circadian rhythms and their associated disorders.

1.2.6.2 Skin cells

The identification of the molecular clock machinery in isolated skin cells has been widely demonstrated. Fibroblasts (Fig. 8) are one of the first peripheral cell lines where a cell-autonomous and self-sustained circadian rhythm has been observed. The cultivation and genetic manipulation of fibroblast are easy to perform and these are some of the main reasons why fibroblasts are a good model of peripheral oscillator organs (see 6.1 Appendix 1).

Skin cells can be easily obtained from a 2-mm skin punch biopsy. At least two cell lines can be successfully isolated from skin biopsy: fibroblasts and keratinocytes. Also hair root keratinocytes cling to the end of a plucked human hair have been cultivated and used as a genetic tool for circadian studies. One of the main difficulties related to the sampling of hair root keratinocytes is that most plucked hairs do not contain keratinocytes and so scalp biopsy would be necessary to isolate hair root keratinocytes [248].

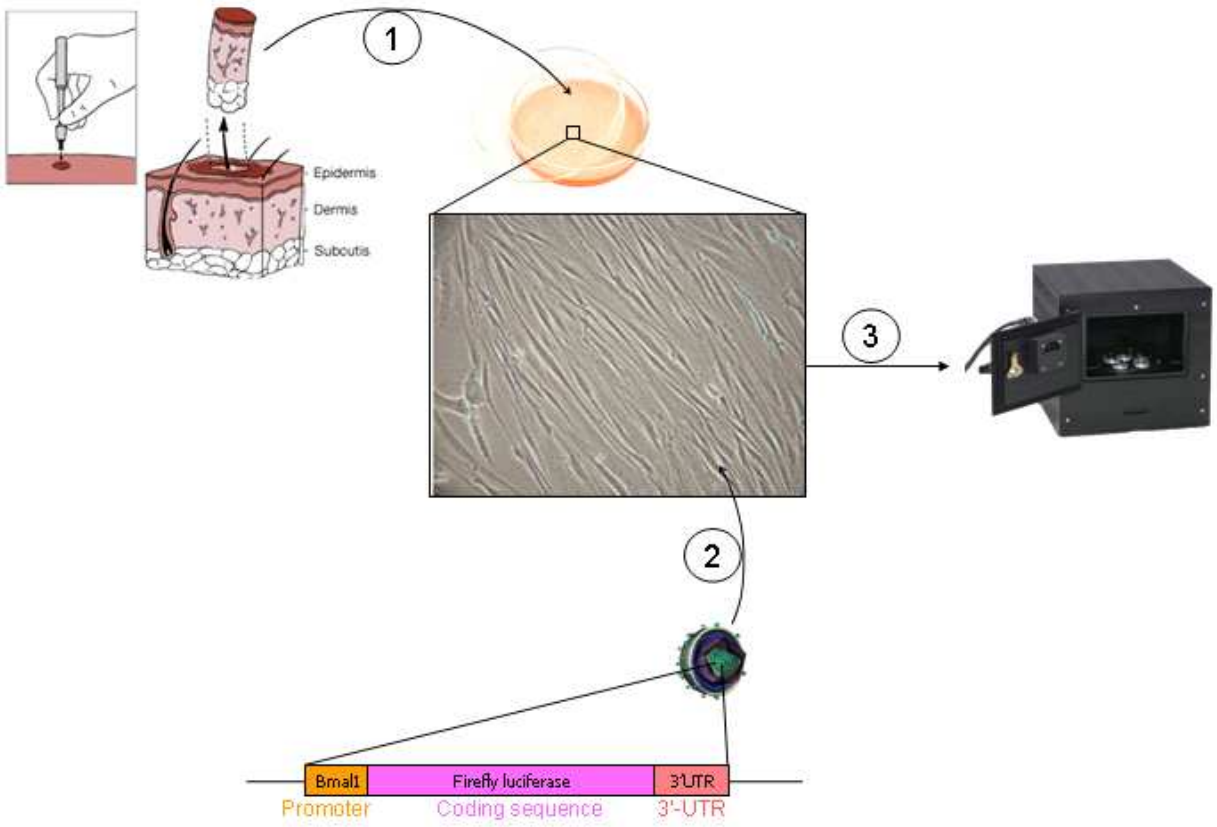

Fig. 8. Protocol to study the circadian rhythms of human skin fibroblasts. Punch skin biopsy is harvested **(1)** and cultivated under sterile conditions. Fibroblasts that grow around the biopsy are isolated, amplified and infected with a circadian reporter **(2)** coding for firefly luciferase under a clock gene promoter, i.e. *Bmal1*. The infected cells are selected and then, after synchronization of circadian rhythms, the light emitted by the cells is measured in the Lumicycle (Actimetrics) or in a home-made device able to detect and measure bioluminescence **(3)**.

1.2.6.3 Signals to synchronize peripheral oscillators in vitro

One of the main differences between SCN slices and other tissues is the damping tendency observed in peripheral oscillators: peripheral cells thus need to be re-synchronized after few days in culture to be able to continue to detect a circadian rhythmicity. The rhythmic damping of peripheral oscillators is predominantly due to phase-desynchronization of individually oscillating cells [247, 292]. To maintain a good rhythmicity (consistent amplitude) it is necessary to resynchronize individually oscillating, but asynchronous, cells. Treatments that increase the coherence of the oscillators reflect the coordination of a phase-shifting efficacy of the treatment. However, different treatments have different strength to activate/inhibit specific signalling pathways, that ultimately result in oscillations of “clockwork” genes of varying amplitude [244].

During a resetting of the SCN clock machinery after a light pulse the expression of *Per* genes is the first to be affected; *Per* is a member of the family of the immediate-early

genes, as well as the transcription factors *c-Fos*, *FosB*, *JunB*; *Per* is implicated in light-mediated phase shifting [293]. The same pattern of Per genes can be observed when peripheral oscillators are synchronized for example through serum shock [246]. Several signals are capable to restart circadian rhythms coherence, increasing *Per* and *Fos* expressions with a similar kinetic observed during SCN circadian rhythms resetting after light pulse [246]. One of the classical methods used to synchronize peripheral oscillators is serum shock, that consists in the incubation of the cells in a high percentage horse serum medium for 1-2 hours [246].

Not only serum shock is able to induce the expression of the immediate-early gene *Per1* followed by the restoration of circadian rhythmicity, but also single substances and other molecules contained in the serum. These substances include: activators of adenylate cyclase (forskolin) [294], activator of protein kinase C phorbol-12-myristate-13-acetate (PMA) [295], angiotensin II [296], calcium ionophore (calcimycin), fibroblast growth factor, the hormone insulin, three activators of receptor tyrosine kinases [220], the activation of the protein kinase C-mitogen-activated protein kinase cascade and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) phosphorylation [297] entothelin-1 [298] and prostaglandin E2 [299].

Another signal that induces the synchronization of cellular circadian rhythms is the activation of glucocorticoid receptor, as it is demonstrated by the use of dexamethasone (an analog of glucocorticoid receptor) shock [220, 300].

Even the simple changing of medium with fresh medium is able to induce coherence on cellular circadian rhythms and probably the key step is the restorement of glucose levels [230]. The medium change does not induce a rapid increase in *Per* genes expression, in contrast with other synchronising agents, suggesting a quite different manner for the induction of the synchronization. The glucose-synchronization is dependent on increasing RNA expression and protein synthesis [230] and it is mediated by the specif glucose-responsive immediate-early genes such as *transforming growth factor ß-inducible early gene 1* (*Tieg1*) and *vitamin D3 up-regulated protein 1* (*Vdup1*). Probably the action of TIEG1 as synchronizing agent sensitive to glucose levels is due to its binding to *Sp1* sequence [301] present near the transcription initiation sites of *Per1* and *Bmal1* genes [302-303], whereas VDUP1, through its negative regulation of thioredoxin, might inactivate CLOCK-BMAL1 [304] and hence reduce the transcription of *Per1* and *Per2* genes.

The synchronization of circadian rhythms mediated through different signals can influence circadian parameters in different ways: indeed, PMA synchronization lengthens the period

length, the synchronization with serum shock and forskolin lead to a later phase of the rhythms, while dexamethasone, 0.001% ethanol and 0.1% dimethyl sulfoxide (DMSO) treatments lead to an earlier phase of the rhythms. The most effective treatments in inducing higher amplitude of the rhythms are dexamethasone, forskolin and serum shock. Cellular circadian rhythms can be sensitive to different substances that affect the same pathway; for example, forskolin, 8-bromo-cAMP (a potent protein kinase A activator), fibroblast growth factor (factors for the receptor tyrosine kinase–mediated mitogen-activated protein kinase pathway), and endotheli-1 are factors that activate CREB phosphorylation, but forskolin is the most efficient in increasing the amplitude of *mPer2::luc* Rat-1 cells [305].

Temperature is another factor that is able to entrain the circadian rhythms of peripheral oscillators. Indeed, cycles of the environmental temperature of about 4℃ are able to sustain fibroblast circadian rhythms [223]. Interestingly, a reverse clock genes phase is observed between cells that have an opposite temperature cycles (e. g. 33:37 versus 37:33). Mammals have a CBT range of 1 to 4℃ [306] and this cycle is able to sustain clock genes circadian rhythms in peripheral oscillators [223].

Even if synchronizing signals *in vitro* may play a role as zeitgeber also *in vivo*, none of the time cues controlled by the SCN is exclusive in entraining peripheral oscillators. Rather, this task may be accomplished via multiple redundant mechanisms, some of which are indirect.

1.2.6.4 Temperature compensation

Temperature compensation is one characteristic that defines circadian rhythms that means that the cycles run with the same period within a physiological range of temperatures [307]. The mechanisms of temperature compensation are still unclear.

The parameter that defines temperature compensation is Q_{10}, that is $(T_2/T_1)^{(10/\theta^1-\theta^2)}$ where T is circadian period length and θ is temperature. Cells are temperature compensated when Q_{10} is around 1.8 and 1.2, whereas the normal biological reactions have a Q_{10} of 2 or 3, i.e. cell cycle [308]. Temperature compensation was demonstrated in several mammals tissues [309], but in nonneuronal tissues the damping was stronger than in SCN [243]. Rat-1 cells in a temperature range between 28.5 and 36.5℃ have a Q_{10} of about 0.9 and period length shortens with temperature decrease [244]. NIH 3T3 have a Q_{10} of about 0.9 in a temperature range between 33 and 42℃ [308]. Moreover, in this cell line the accumulation of clock gene proteins is greater at higher temperatures [308].

On the contrary, temperature over-compensation is the situation when at lower temperatures the clock oscillates faster.
The increase in damping rate at higher temperature is probably due to a damping of the cellular pacemaker itself and not due to desynchronization of cells within the population [244].

1.3 AGEING AND THE CIRCADIAN OSCILLATOR

As the body ages, the coordination of circadian rhythms deteriorates in universal fashion: the circadian organization of the sleep-wake cycle is disrupted and with it most other physiological manifestations of the circadian clock become less pronounced [310] (see also 6.2 Appendix 2). In situations of pathological ageing such as dementia and Huntington's and Parkinson's Diseases, initial disease stages are marked by abnormal daily behavior and later stages are correlated with almost total loss of circadian function [311-312]. An understanding of this deterioration could therefore be of great assistance in increasing the quality of life for elderly individuals. Incorrectly, many of these disturbances are ascribed simply to "sleep difficulties": in western countries one in five elderly people reports taking a sleep medication regularly [313]. While these may provide a momentary alleviation of fatigue, underlying causes remain unaddressed, leading to a chronic dependence upon these substances.

1.3.1 Involvement of circadian system in ageing

There are several links that connect the impairment circadian system with the reduction of life span and cancer and other age-related pathologies [314-315]. The disruption of the circadian system, in the arrythmic *Bmal1* deficient mice, highly reduced life span of the animals; from the adult age, mice lacking *Bmal1* have a lower body weight due to a reduced mass of adipose and muscle tissue. These mice showed earlier in age symptoms of age-related pathologies such as sarcopenia, progressive cornea inflammation and cataracts, hair loss. The early ageing phenotype correlated in these mice with increased levels of oxidative stress accumulation in kidney, heart and spleen [316]. An imbalance between circadian system and oxidative stress production and scavenger system may contribute to and/or complicate pathogenesis and aging.
Recent findings on the redox-sensitive input into the core oscillator via modulation of NPAS2/BMAL1 or CLOCK/BMAL1 heterodimer binding to DNA indicate a direct influence of cellular redox balance, including oxidative stress, on the circadian clock [317]. An

example of the interconnections between the circadian system and cellular redox state is the nicotinamide adenine dinucleotide (NAD) ± dependent protein SIRTUIN (SIRT1) [318]. SIRT1 is a histone deacetylase required for the circadian transcription of *Bmal1*, *Rorγ*, *Per2*, and *Cry1* genes; in addition SIRT1 bound to the CLOCK:BMAL1 complex in a circadian fashion and promoted the deacetylation and subsequent degradation of PER2 [319] and the deacetylation of BMAL1 at Lys537 [320]. SIRT1 is regulated in a circadian manner and its deregulation leads to significant disturbances in the circadian cycle due to acetylation of histone 3 (H3) and BMAL1 [320].

In another study CLOCK:SIRT1 complex was shown to regulate the circadian levels of NAD^+ by regulating the circadian expression of the enzyme nicotinamide phophoribosyltransferase (NAMPT), the rate-limiting step in the NAD+ salvage pathway [321]; the inhibition of NAMPT promoted the oscillation of the clock gene *Per2* by releasing CLOCK:BMAL1 from SIRT1 suppression [322].

Accumulated evidence suggests that SIRTs are involved in promoting longevity [323-326] in several organisms. The first evidence of a link between longevity and SIRTs was found in C. elegans [327] and yeast [328]; for example in *Sir2* mutant yeasts rDNA circles are accumulated due to a disregulation in the recombination processes [329-330]. In glucose starvation regimen the yeast mother cell longevity is extended in a Sir2-dependent manner [331].

SIRT1 deacetylates non-histone proteins such as the p53 tumour suppressor protein, alpha-tubulin and forkhead transcription factors to mediate diverse biological processes including metabolism, cell motility and cancer [325]. In mammalian cells, SIRT1 has been shown to down-regulate stress-induced p53 and FoxO pathways for apoptosis, thus favoring survival [324, 332]. In the absence of SIRT1 p53 is hyperacetylated [333] and when the hyperacetylation of p53 is accompaigned by DNA damage cells undergo apoptosis.

Treatment of yeast cells with resveratrol, an agonist of Sir2 extends yeast mother cell longevity in a Sir2-dependent manner [334]. Experiments in *C. elegans* and *Drosophila* [335-336] suggest that resveratrol (and related polyphenolic compounds) can increase longevity in very diverse organisms. In human cell culture treatment of cells with resveratrol can increase longevity by modulating SIRT1 activity on p53 and tumor necrosis factor (TNF) alpha-induced apoptosis [337].

The majority of SIRT1 null animals succumb to developmental defects during early postnatal period maybe due to cardiac defects [333]. SIRT1 deficient mice report a failure

to thrive, abnormal retinal histology with rosette formation, sporadic exencephaly, and cardiac septal and valvular abnormalities [333]; moreover, lung or pancreatic defects were reported in these animals [338].

1.3.2 Circadian behaviour modifications

The most common symptom of circadian rhythm disruption during ageing is the impairment of the sleep-wake cycle (see also 6.2 Appendix 2). Aged people show a reduced amplitude of the activity compared to young people, which is marked during evening hours [339], whilst time spent awake during the night increases [340]. Moreover, sleep hygiene is serious compromise in elderly: a reduction in the promotion of sleep by the circadian pacemaker during the biological night in combination with a reduced homeostatic pressure for sleep may be causes for the observed earlier awakening compared to the circadian phase, loss of sleep consolidation specially in NREM sleep [341] and increased sleep latency [342-343]. Alterations in sleep homeostasis are confirmed by the observation of a decreasing in slow-wave sleep across the life span [344]. However, the imposition of a regular routine of mealtime and exercise may improve sleep hygiene and the amplitude of the sleep-wake cycle [345-346].

The phase of entrainment of the activity changes with age: during the first phase of life the chronotype is very early and tends to delay, wereas from the puberty the tendency inverts for the rest of the life span [347]. Females, that mature earlier, present this trend of advanced chronotype compared to males; however, from the menopause females have the same chronotype as age-matched males [348]. Moreover the correlation between circadian phase and chronotype with circadian period length present in young adults [90] are lost analysing older subjects [349].

A hypothesis for the sleep-wake cycle and chronotype alterations is a lower sensitivity in the entrainment of light during elderly [350-351]. Indeed, in mammals the circadian system entrains mainly to the light signals that enter the eyes and are capted and translated into biological signal by the retina photoreceptors. Then the retina then sends these signals to the SCN [78]. However, within elderly, many changes in the eye physiology occur and may be the reason of impaired entraining properties in aged people. Elderly have a lower transmittance of the crystalline lens also due to the yellowing of the lens, especially at higher visible wavelength, impairing the possibility to stimulate opsins, such as melanopsin that peaks around 460 nm [352-353]. Pupil area is decreased in elderly, leading to the senile miosis, reducing further the light that enters in eyes [354]. Moreover, a poor light

quality and quantity of artificial light [355] for hospitalized elder people may exacerbate the lower capacity of entrainment of older people. From the early 1990s during cataract surgery the lens were replaced with yellowish blue-blocking intraocular lens [356]. This particular lens diminished the possibility for the opsin, in particular melanopsin, to entrain circadian rhythms of the SCN. Nowadays, the use of UV-only blocking intraocular lens are favoured for the replacing of cataract and the improve of nocturnal sleep and reduction of daytime sleepiness suggests an increasing of the amplitude of the patient's circadian rhythms [357].

To counteract the changes in the entrainment capacity and eyes physiology that occur during ageing valid treatments are the combination of melatonin and light treatment; whereas light therapy alone attenuated cognitive deterioration, ameliorated depressive symptoms, attenuated the increase in functional limitations and melatonin alone shortened sleep onset latency, increased sleep duration, the combination of the two therapies attenuated aggressive behaviour, increased sleep efficiency and improved nocturnal restlessness [358-359].

1.3.3 Hormones changes during ageing

In adult animals the immune system is able to influence circadian rhythms: functional receptors for IFN-γ (IFN-γ) are found in the ventral part of the SCN [360-361] and present a daily rhythm [362] and the intracerebroventricular injection of the pro-inflammatory cytokine IFN-γ affects locomotor activity [363] and sleep [364]. Also the cytokines TNF-α and IL-1β are shown to modify some clock genes expression *in vitro* and *in vivo* in animals; the effect of TNF-α on clock genes is also present *in vivo* in the SCN and probably it is the mechanism that leads to a decrease activity mice during the dark period. Moreover, TNF-α is shown to inhibit CLOCK-BMAL1-induced activation of E-box regulatory element-dependent clock gene promoters [365].

During ageing functions of the immune system change, with an expansion of memory T cells [366] and increasing levels of pro-inflammatory cytokines, also in the brain [367] which may indicate chronic low-grade inflammation [368]. A correlation between some inflammatory mediators, such as IFN-γ, and longevity was found [369-371], and in non-human primates IFN-γ levels were found to correlate with rhythm parameters of locomotor activity and body temperature and are characteristic of aging [369].

In addition, altered responsiveness of SCN neurons to i.c.v. injections of cytokines with aging indicates an age-dependent dysfunction in the interaction between immune factors and biological clock [372-374].

Ageing leads also to an impairment of the the hypothalamic-adrenal-pituitary (HPA) axis, revealed by a decreased amplitude of cortisol levels especially during the evening [375] and a reduced HPA sensitivity to steroid feedback [376-377]. When ageing accompaigned dementia, the adrenal production of dehydroepiandrosterone and of its sulfate was even stronger [375].

Some pathology associated with ageing, such as atherosclerosis and hypertension, may be due to a reduced production of endothelium-derived NO that is a critical regulator of cardiovascular homeostasis. The reduced NO production may affect also circadian rhythmicity, since the presence of NO donor induced *Per2* gene expression. In elder animals daytime NOS activity is reduced, as well as clock gene expression; this situation is mimicked by blocking NO production in young animals. Probably NO increases *Per* expression by acting through cAMP response element-dependent and the E-box enhancer element-dependent pathways. Another link between NO presence and the modulation of circadian rhythm is given by the finding that NO donors caused the S-nitrosylation of BMAL1 protein and thus increased its stability [378]. *Bmal1* knock-out mice have a disrupted blood pressure variation and heart rate [379], but the blood pressure was restored in the presence of NO donor [378].

1.3.4 Alterations of circadian parameters and entrainment

Althout animal studies and SCN slices show a shortening in period of sleep-wake cycle during ageing [380-381], human studies revealed no age-related reduction of period length considering CBT of sighted and blind subjects [342], melatonin and cortisol rhythms [260]. However, an increasing number of data demonstrate an influence of ageing on circadian rhythms amplitude. For example, CBT amplitude [138, 342], cortisol and growth hormone [382] amplitudes are affected by ageing in human.

During ageing the amplitude of melatonin production and secretion decreases [312] (Fig. 9); in rat hypothalamus, this decreasing is paralled by the decreasing of melatonin receptors (MTs) [383]; the same finding was shown in human SCN [384].

Also in animals a marked decrease of circadian rhythms amplitude is observed [385], such as in rodent activity [386] and SCN firing rate [387] (see also 6.2 Appendix 2). Interestingly, in addition to a decreased amplitude, the variability in the circadian waveform

of dispersed SCN cells is increased in aged animals [388], implying that aging could either disrupt coupling between SCN pacemaker cells or their output, or cause deterioration of the pacemaking properties of SCN cells [389-390].
Another circadian parameter that is influenced by ageing is the phase: ageing leads to a phase delay peak of melatonin compared to wake-up time [391] and an advanced time of awakening, CBT and plasma cortisol [392-393]. Moreover animal studies revealed an age-related PVN, pineal gland and kidney cells phase advance [381].

Ageing reduces the phase shifting properties of light: in human a reduced phase shifting of blue-light was observed [394-395], whereas in animals the reentrainment to a new light-dark schedule takes more days [386, 396]. Peripheral organs show a stronger impairment to the resynchronization to a new light-dark schedule compared to SCN, as it was demonstrated in aged rats [397].

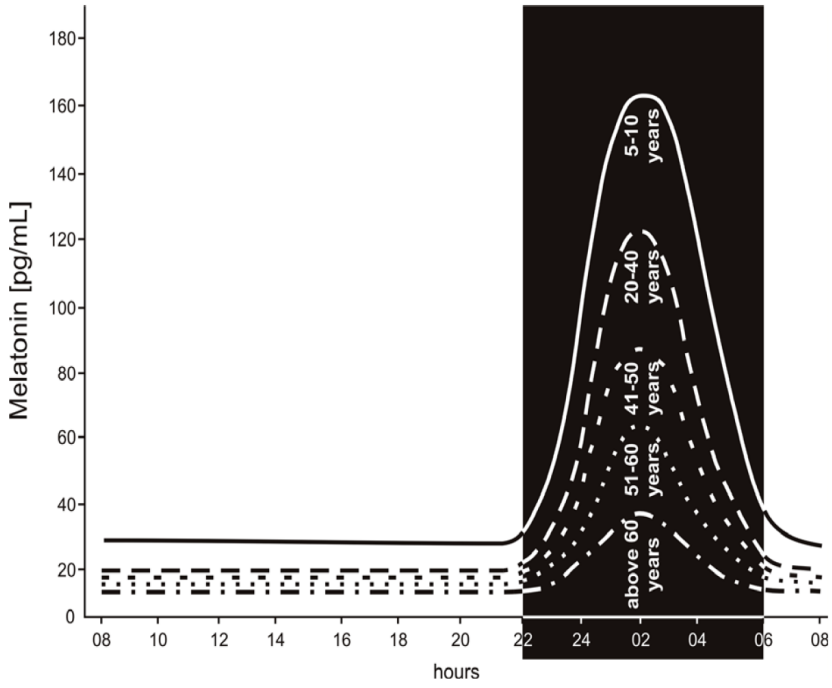

Fig. 9. Hypothetical example of the reduction of a circadian marker during ageing. Melatonin secretion shows great variability within individuals; normally melatonin appears in humans from the 6th-8th week of life and reaches the maximum between the 4th and the 7th year of age. However, already around 20-40 years of age the amplitude of melatonin secretion, as well as other circadian markers, decreases. At around 70 years of age the nocturnal peak of melatonin secretion is barely detectable. Throughout ageing melatonin secretion levels can extremely change up to 100 fold. Black bar represents the night period. (From [398]).

1.3.5 SCN influences on age-related circadian disorders

Many findings indicate that changes in the SCN underlie some of the sleep disturbances among elderly people [342-343, 399] (see also 6.2 Appendix 2). In post-mortem analyses of human brain, many neuropeptide cycles were impaired during elderly, such in the case of vasopressin that is is present with a marked decreased amplitude rhythm in aged people [399].

During ageing dramatic changes in SCN electrophysiological activity occur. The period length of the SCN cells of aged animals appear to be shorten compared to young animals [381]. A long-term, single-cell recording of mouse SCN neurons showed that ageing induced a marked decrease in the amplitude of impulse activity and an increase in variability in the circadian waveform [388]. Similarly, the measurement of the ventrolateral part of the SCN revealed that the the amplitude of the spontaneous neuronal firing rate was reduced, due to a higher night activity. This effect is owed to a decreasment in the spontaneous GABAergic synaptic activity during both day and night [400], where GABAergic activity does not show a rhythm, unlike in the dorsal part [401]. $GABA_A$-α3 subunit does not show an age different expression in mouse SCN [402], as well as the GABA-synthesizing enzyme glutamic acid decarboxylase isoform 67 (GAD67) RNA and protein levels [402]; however, the number of GAD67 positive neurons are decreased with ageing of the animal in the areas with both dense and sparse retinal afferences [402]. Similar results are found also in hamster, where the SCN spontaneous firing rate was lower in aged animals in the subjective day [390].

The expression of VIP is reduced during ageing in hamsters and rats and this reduction may contribute to the alteration in the entrainment and attenuation of phase resetting that can be observed during ageing [403-404]. Also the diurnal amount of the receptor VPAC2, VIP and PACAP receptor [405-406], are reduced in aged rats [404]. Moreover, levels of VIP mRNA in rats have a very narrow rhythm amplitude [407]. As a result, melatonin inhibition on CREB-phosphorylation PACAP-induced is impaired, suggesting a loss of sensitivity of mouse SCN to melatonin [408].

Interestingly, SCN transplant could restore circadian rhythms of aged animals. In hamster the age-related alterations on the circadian system, such as the locomotor activity and the loss of responsiveness to the phase shifting or entraining substances can be reversed by implanting old animals with fetal SCN tissue [409-411]. In another accurate study on SCN transplant in aged hamsters, the grafted host behaviour was not completely reversed to the donor behaviour, but in most of the cases the older hamsters showed a coordination between donor and host rhythmic behaviour [412]. The results suggest that the expression of rhythmicity by the grafted SCN may depend on the relative amplitude or strength of signals produced by the host and donor SCN.

In a study conducted on rats, only the half of the grafted animals benefited by the transplant, showing improvements of one or more rhythms (locomotor, drinking,

temperature); the number of VIP-positive cells correlated with the improvement circadian behaviours [413].

1.4 MELATONIN

Melatonin is a hormone synthetised primarily in the central nervous system. The major source of melatonin is the pineal gland, a small pine cone shaped endocrine gland located near the center of the brain, between the two hemispheres, tucked in a groove where the two rounded thalamic bodies join, mainly consisting by pinealocytes, cells that produce and secrete melatonin [414]. Melatonin production was demonstrated to occur also in sites outside the pineal gland such as: retina [415], Harderian gland [416], gastrointestinal tract [417], testes [418], human lymphocytes [419] and melatonin synthesis enzymes are found expressed in skin tissues [420]. Melatonin synthesis and secretion occur only during the dark period of the day in all the species; moreover, in photoperiodic species, melatonin amplitude profile correlates with the lenth of the night [421].

1.4.1 Melatonin synthesis and degradation

Melatonin, a small indoleamine, is synthetised throught a cascade of enzymatic reactions from tryptophan (Fig. 10). The rate limiting in melatonin production is the enzyme arylalkylamine N-acetyl transferase (AA-NAT), whose levels are 7-150 fold higher in the night than in the day. Also serotonin, the substrate of AA-NAT, may play a role in the regulation of melatonin production.
AA-NAT levels in human are regulated primarily at post-transcriptional levels, whereas in rodents the regulation appears to be related to a cAMP-dependent phosporylation of a transcription factor that binds to the AA-NAT promoter. Rapid decline in activity with light treatment at night appears to depend on proteasomal proteolysis.

The signal that regulates the presence of AA-NAT comes from the retina (Fig. 10); indeed, retina photoreceptors convert light and darkness into signals that are sent directly to the SCN through the main pathway, the retino-hypothalamic tract (RHT) [422]. From the SCN, neuronal projections make synaptic connections in the PVN of the hypothalamus descending onward through the medial forebrain bundle to the intermediolateral cell column of the spinal cord from where preganglionic fibers reach the superior cervical ganglia [423]. Sympathetic postganglionic noradrenergic fibers from the superior cervical ganglia innervate the pineal gland through the *nervi conarii* [424]. Thus, production of

pineal melatonin occurs in response to noradrenergic stimulation which produces a cascade of biochemical events within the pinealocyte. Noradrenaline release from the sympathetic nerves that innervate the pineal gland is normally high at night and low during the day [425]. In most species, noradrenaline interacts with both $beta_1$- and $alpha_1$-adrenergic receptors present in the pineal gland [426]. In the rat pinealocyte, stimulation of adrenergic receptors induces a rise in adenylate cyclase and cAMP signaling pathway that stimulates AA-NAT expression and phosphorylation via protein kinase A, which also allows AA-NAT to be stabilized by binding of 14-3-3 proteins [427-428]. The nocturnal exposure to bright light suppresses melatonin production immediately by degradation of pineal AANAT [429]. Simultaneous activation of $alpha_1$-receptors potentiates the effects mediated through the $beta_1$-receptors [430]. Locally in the pineal gland, the rhythmic melatonin synthesis is ensured by the oscillating cAMP-dependent transcriptional control mechanism [431].

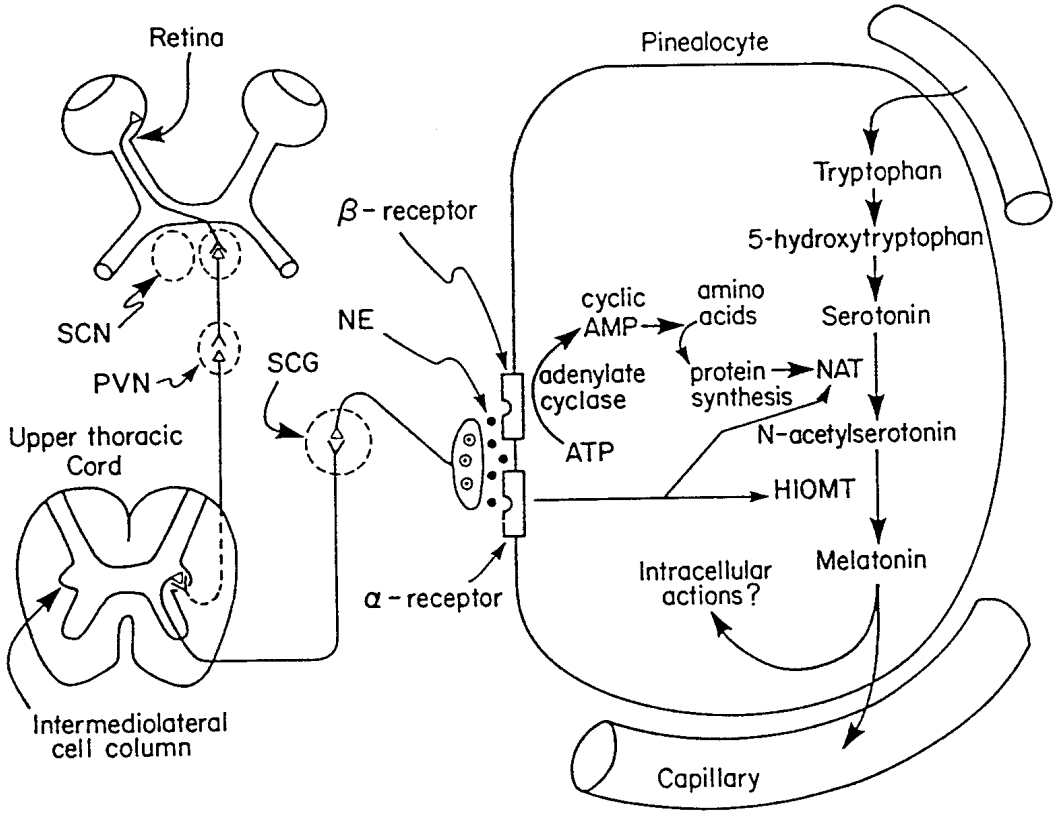

Fig. 10. Melatonin production and secretion process. Melatonin is synthesised from tryptophan through several enzymatic steps. The enzyme N-acetyltransferase (NAT) is the limiting step of the enzymatic melatonin synthesis cascade. The major site of melatonin production is the pinealocytes in the pineal gland. The pineal NAT is regulated, through a multi-synaptic pathway, by light condition. Light information from the retina are sent to the master clock from the retina, then to the paraventricular nucleus (PVN) of the hypothalamus, descending onward through the medial forebrain bundle to the intermediolateral cell column of the spinal cord from where preganglionic fibers reach the superior cervical ganglia (SCG). Sympathetic postganglionic noradrenergic fibers from the SCG innervate the pineal gland through the nervi conarii. Through $beta_1$- and $alpha_1$-adrenergic receptors present in the pineal gland nocturnal noradrenerdic stimuli activate NAT through a signalling pathway that involves the increasing of cyclic adenosine 3´,5´-monophosphate production by adenylate cyclase. (From [432]).

Melatonin half-life is short, between 20 and 60 minutes and is degraded by 6-hydroxylation to 6-hydroxymelatonin, which is sulfated to 6-sulfatoxymelatonin and excreted in urine. Minor degradation pathways are by *O*-demethylation to *N*-acetyl-5-hydroxytryptamine, which is further conjugated to its sulfate and its glucuronide and the deacetylation to 5-methoxytryptamine [433]. The principal enzymes involved in melatonin metabolism are: CYP1A1 [434], CYP1A2 [435] and CYP1B1 [436] that is an ubiquitous extrahepatic CYP melatonin 6-hydroxylase enzyme, demonstrating that the hepatic metabolism may not be the only pathway for melatonin clearance [420].

1.4.2 Melatonin binding sites

From the discovery of melatonin many efforts were done to unravel the nature of melatonin binding sites, their function and signaling pathway [218]. So far, several binding sites were discovered.

Melatonin can bind calmodulin [437], calreticulin [438] or tubulin [439-440] and so preventing the calcium ion binding [437]. This action may be the responsible for the antiproliferative effects of melatonin on breast cancer [441].

Melatonin was reported to be a free radical scavenger by direct chemical reaction to oxidative species [20], as well as an increase of the production of enzymes to counteract the oxidative species [442].

Melatonin has the capability to bind the retinoid related orphan nuclear hormone receptor (RZR/ROR) [443]. The function of melatonin binding to the orphan receptor is not totally understood, even if melatonin activation of RZR/ROR was shown to induce the repression of 5-lipooxygenase mRNA expression in human B cell lines [444] and regulate IL-2 and IL-6 production by human mononuclear immune cells [445-446].

In mammals melatonin act mainly through three types of receptor: MT1, MT2 and MT3. MT1 and MT2 are G-coupled 7 transmembrane receptors [447]. MT1 is 350 aminoacid, whereas MT2 is 362 aminoacid long; their molecular weight is around 39-40 KDa [448-449]. Both MT1 and MT2 are high affinity receptors, even if MT2 (Kd = 160 pmol/l) has a lower affinity for ^{125}I-melatonin compared to MT1 (Kd = 20-40 pmol/l) [450].

MT1 and MT2 melatonin receptors have potential glycosylation sites in their N-terminus and phosphorylation sites for several kinases such as protein kinase C, casein kinase 1 and 2 and protein kinase A [451]; these sites may participate to the regulation of the receptor functions.

In MT1 and MT2 the intracellular loop II is slightly different compared to the other members of the G-protein coupled receptor and the DRY or ERY motif is substituted with the NRY [452]; this region is involved in the receptor trafficking and cell signalling [453]. The disulfide bond between Cys 113 and Cys 190 is essential for the high affinity binding of MT2 and possibly of MT1 [454].

MT1 and MT2 protein and RNA were reported to have a circadian regulation in their expression and well as regulation by light and melatonin concentration. For example, in the rodent SCN and pars tuberalis (PT) MT1 expression is circadian, with higher levels during daytime; nocturnal light administration increases melatonin levels [455-456], leading to a downregulation of the receptor by melatonin [457-458]. Indeed, if melatonin

suppression by constant light or pinealectomy is followed by exogenous melatonin administration the receptor densities in PT decrease [459].

However, it should be mentioned that the expression levels of MTs in the murine SCN are very low during the day, when melatonin levels are low, and high at night, when melatonin concentrations peak [460]. Hence the increases in MT concentration parallels the increases in the level of its ligand, suggesting that the regulation of the receptor level and sensitivity by melatonin is somewhat more complex than earlier studies had concluded and may depend upon the tissue and the species considered. Paradoxically, a light pulse delivered during the subjective night (expected to halt melatonin secretion) decreased the specific binding of radiolabeled melatonin to MT1 receptors in the rat SCN [456].

Estradiol was shown to regulate MT1 mRNA expression in the rat hypothalamus [461], as well as ageing [383, 462]. During ageing MT1 levels in human SCN and cortex decrease [463], although an increase in MT1 and MT2 protein was also reported in hippocampus and retina, and hippocampus and cortex respectively [464].

MT3 was later characterized as quinine reductase 2 [465]: this detoxification enzyme belongs to a group of reductases that participate in the protection against oxidative stress by preventing electron transfer reactions of quinones.

MT3 shows fast kinetics of association/dissociation and a characteristic peak of melatonin binding at 4℃; this peak is not present in the other receptors that show higher binding rates at rising temperatures [466]. The functions of MT3 are still not clear. Moreover, MT3 affinity is higher for N-acetylserotonin than melatonin, and melatonin to serotonin, and appears to couple to stimulation of phosphoinositide turnover [467].

In rats and hamster low affinity binding sites (Kd about 10 nM) were demonstrated in the preoptic area of the hypothalamus and in the medulla-pons [383]; however, the proteins involved in this activity are still unidentified.

1.4.3 MT1 and MT2 signal transduction pathway

MT1 and MT2 were demonstrated to be coupled and to activate different G-proteins, such as $G_{i1\text{-}3}$, possibly Gs and $G_q/_{11}/_{14}$. A coupling of MT1 and MT2 with the promiscuous G_{16} was demonstrated in COS-7 cells [468-469]. In several *in vitro* studies (NIH, HEK, CHO) as well as in recombinant cell lines one of the major pathway that is activated by MT1 and MT2 is the inhibition of forskolin-stimulated cAMP accumulation through Gi activation, by inhibiting the protein kinase A (PKA) activity and phosphorylation of the transcription factor

cAMP-responsive element binding protein (CREB) [470-471]. This is the same pathway activated by melatonin that was shown to mediate melatonin phase shifting properties in animals [472]. However, the signal transduction pathways for MTs appear to vary among different tissues and cell types [473-474].

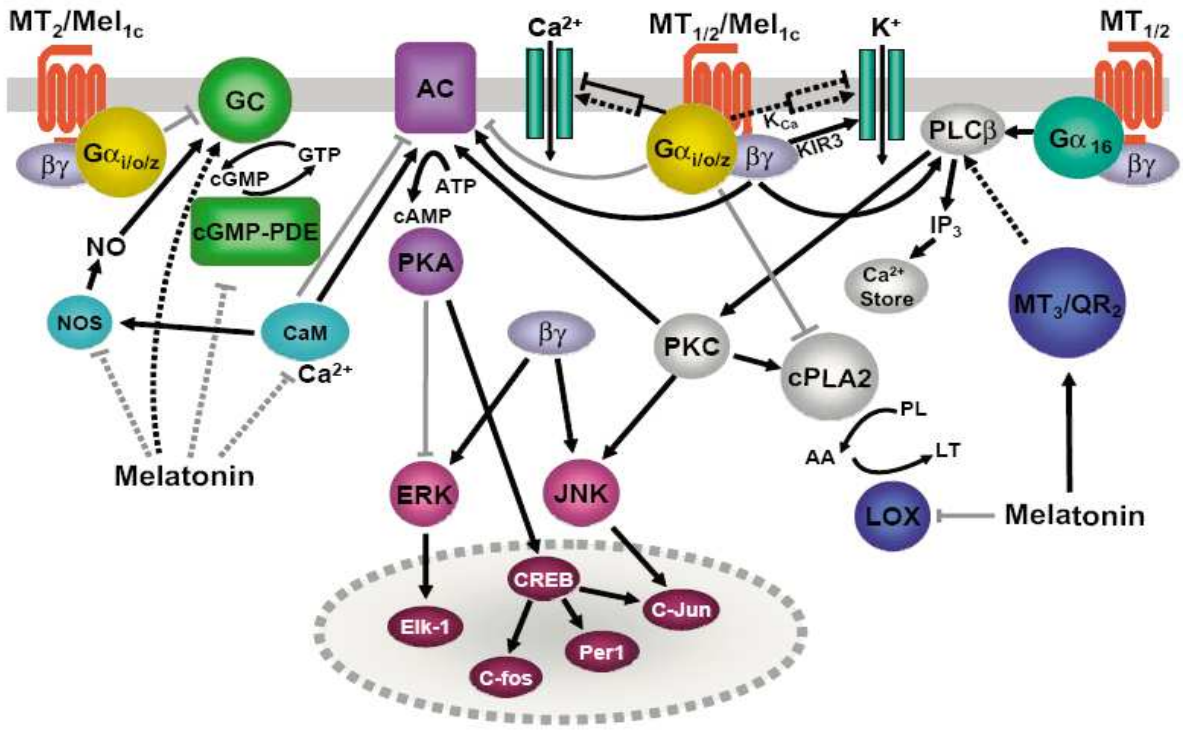

Fig. 11. Schematic representation of melatonin signalling pathway. Melatonin receptors MT1 and MT2 are coupled to several heterotrimeric G proteins, activating specific downstream pathways. Dashed lines indicate that the role of intermediate proteins has not yet been elucidated. AA = Arachidonic acid; AC = adenylyl cyclase; CaM = calmodulin; CREB = cAMP-responsive element binding protein; cGMP-PDE = cGMP phosphodiesterase; cPLA2 = cytosolic phospholipase A2; ERK = extracellular signal-related kinase; GC = guanylyl cyclase; JNK = c-jun NH2-terminal kinase; KCa = Ca2+-dependent K+ channels; KIR = G protein-gated inwardly rectifying potassium channels; LOX = lipooxygenase; LT = leukotrienes; NO = nitric oxide; NOS = nitric oxide synthase; PKA = protein kinase A; PKC = protein kinase C; PL = phospholipids; PLC = phospholipase Cß; QR2 = quinine reductase 2. (From[475]).

1.4.3.1 MT1

MT1 receptor can be coupled to several G-proteins such as i, o, q, z, 12, 13, 14, 16 [475-476]. The coupling with G_{i2} and G_{i3} proteins mediate adenylyl cyclase (AC) inhibition and as a consequence inhibition of forskolin stimulated cAMP formation, PKA activity and phosphorylation of CREB [470], while the coupling with $G_{q/11}$ protein increases phospholipase C (PLC) activity [477-478]. The β and γ subunit of G-protein potentiate ATP or Prostaglandin $F_{2\alpha}$-induced release of arachidonate and hydrolysis of phosphoinosite [478-479].

Other pathways may be activated by MT1; in MT1 transfected COS-7 and neuroblastoma SH-SY5Y melatonin causes stimulation of cAMP; the mechanism is still not clear but it is probably mediated through a calcium-calmodulin signal transduction pathway and c-Jun N-terminal kinase activation [480-481].

The activation of MT1 by melatonin is also able to induce a transient elevation of cytosolic Ca^{2+} and inositol phosphate accumulation [477-478] and activate the mitogen-activated protein kinase and ERK kinase 1/2 (MEK172) and extracellular signal-regulated kinases 1/2 (ERK1/2) probably leading to induction of synthesis of filamentous structures non-neuronal tissues [482].

Moreover, it also regulates functional responses of melatonin in ion channels. For example, it was shown that vasoconstriction of rat arteries is mediated by melatonin-induced blockage of calcium activated potassium channels [483] and the activation of the inward rectifier potassium channel KIR3, which leads to inhibition of neuronal firing in the SCN [484].

1.4.3.2 MT2

Similar to the second messenger pathways of the MT1 receptor, activation of the MT2 receptor also inhibits forskolin stimulated cAMP formation [471]. Moreover MT2 mediates the increase in phosphoinositide production and the inhibition of cGMP formation [485] and cGMP pathway [486].

Another pathway activated by MT2 is the stimulation of phospholipase C and diacylglycerol-mediated activation of protein kinase C that was shown linked to the phase shifting properties of melatonin in several studies using rat SCN slices [487-488].

1.4.3.3 AC

Apart from the classical inhibition of AC due to melatonin activation of G_i, more complicated pathways may involve melatonin in the activation/repression of AC.

There are 9 different isoforms of AC and every isozyme exhibits different sensibility to $G\alpha_i$, $G\beta\gamma$ and Ca^{2+}/calmodulin. Indeed, type 1-6 AC are inhibited by $G\alpha_{i1-3}$ and $G\alpha_z$, type 1 is inhibited by $Go_{A/B}$ and $G\beta\gamma$. On the other hand $G\beta\gamma$ and activated $G\alpha_s$ or protein kinase C (PKC) stimulate AC 2, 4 and 6 [489]. This activation is observed in the VIP stimulated cAMP accumulation in human lymphocytes; since human lympocytes express $G\alpha_{i1-3}$, $G\alpha_z$, and $G\alpha_s$ [490] and MTs [491], if type 2 or 4 AC is also present in lymphocytes, it is entirely possible for MTs to enhance the VIP-induced response via the $G\beta\gamma$ released from G_i or G_z.

Ca^{2+}/calmodulin can inhibit AC 5 and 6 isoforms but activates type 1, 3 and 8 [492]; since AC 5 and 6 are expressed in human lymphocytes [493] and melatonin antagonizes Ca^{2+}/calmodulin function [494], the potentiation of VIP induced cAMP accumulation may be due to the attenuation of the inhibition of Ca^{2+}/calmodulin by melatonin. However, the binding of melatonin with Ca^{2+}/calmodulin may inhibit the activation of AC types 1, 3 and 8. The overall effect on AC activity by the binding of melatonin to calmodulin will be determined by the balance of its inhibitory and activatory effects on AC.

Another way by which melatonin may stimulate AC type 1-8 is through the activation of PKC [492].

Moreover, melatonin block of the forskolin-stimulated increased levels of *c-fos and junB* mRNA levels [495] may be due to the inhibition of cAMP production by melatonin, that as a consequence leads to the block of the activation of PKA [496] and CREB [497]. This effect is important especially considering the ovine PT and the seasonal changes which are regulated by melatonin [498].

1.4.3.4 PLC

PLC are enzymes that catalize the convertion of phospholipids to diacylglicerol and inositol triphosphate. However, only the isoform PLCβ is activated by G protein; in particular melatonin activates PLCβ through the activation of MT1 and MT2 that then $G\alpha_{16}$ [499] or $G\beta\gamma$ subunits, as demonstrated in MT1 and MT2 tranfected CHO cells [500] and rat SCN slices [487]. In a study on MT1 transfected HEK cells the activation of PLCβ was possible if Gq was coactivated [478].

The expression levels of MTs in the SCN and PT vary throughout the light/dark cycle, which are in part regulated by melatonin itself [459]. Receptor levels are sensitive to PKC [501] and as PKC activity in the SCN is responsive to melatonin [487] it is possible that the PLC/PKC pathway is employed to modulate the sensitivity of cells to melatonin. This may also occur by melatonin-induced PKC activity being responsible for phosphorylation and desensitization of MTs, as demonstrated in human prostate epithelial cells [457].

1.4.3.5 Phospholipase A2 (PLA2)

PLA2 is a cytosolic enzyme that catalazes the production of arachidonic acid (AA) from phospholipids. In MT1 transfected NIH3T3 cell lines melatonin activates Gi and PKC facilitating the activation of PLA2 stimulated by PGF2α activation of Gq-coupled pathways, with no effect on the basal level of AA secretion [479].

Another effect of melatonin on AA is the inhibition of the metabolism by human platelet cyclo-oxygenase [502] and downregulation of 5-lipoxigenase in human B lymphocytes via the activation of a nuclear melatonin receptor RZR/ROR [444] and of 12-lipoxygenase in the rat pineal gland [503], resulting to an increased levels of AA.

However, melatonin was also shown to inhibit the hormonal-induced AA accumulation and oppose AA-induced physiological responses: melatonin inhibits AA-induced platelet aggregation [504] and thromboxane-B2 production in pancreatic cells [505] and in the rat anterior pituitary melatonin administration downregulates PLA2 expression and AA production [506].

The composition of effectors in a particular cell type may play a key role in the responsiveness of cPLA2 to melatonin.

1.4.3.6 K+ channels and Ca2+ channels

Melatonin acutely depresses the *in vitro* electrical activity of SCN neurons in hypothalamic slices [507] indicating that the modulation of the electrophysiology of neurons contributes to the propagation of melatonin effects. In particular melatonin regulates potassium and calcium channels.

Potassium channels: Potassium currents play a critical role in maintaining the resting membrane potential of cells, the control of neuronal firing rates and the regulation of neurotransmitter release [508].

In SCN neurons and rat cerebellar granule cells melatonin induces an outward potassium current and inhibits an inward cation current and firing rate, through activation of MTs but not via cAMP or PLC arthways [484, 509-510]; indeed, members of the Kir3 potassium channel subfamily, which are widely expressed in the brain, can be activated by $G\beta_i\gamma_2$ via direct interaction, but the $G\alpha_{i1}$ subunit counteracts $G\beta_i\gamma_2$-stimulated Kir3.1 activity [511-512]. It would seem that melatonin regulates Kir3 channels in the brain via a balance between activation and release of $G\alpha$ and $G\beta\gamma$ subunits.

Through its regulation of cAMP and NO/cGMP, melatonin is able to indirectly modulate also the Ca^{2+}-dependent potassium channel family (KCa), responsive to these second messengers calcium [508]. Melatonin, acting via MT1 and MT2 receptors, was indeed been demonstrated to inhibit the large conductance (BKCa) channels through the decrease in the cAMP-protein kinase A pathway that phosphorylates channels and this inhibition potentiates the constriction of vascular smooth muscle [483, 513], although whether this is mediated via $G\beta\gamma$ subunits or a $G\alpha$-coupled pathway is not clear.

Furthermore, melatonin-induced inhibition of the small conductance (SKCa) channels in rat gastric smooth muscle results in the relaxation of pre-induced vasoconstriction [514].

Calcium channels: Two groups of Ca^{2+} channels, voltage-dependent Ca^{2+} channels (VDCCs) and second messenger-operated Ca^{2+} channels (SMOCs), are putatively involved in the melatonin signaling pathways. VDCC channels may be modulated by G protein: L-, N- and P/Q-type VDCCs are inhibited by Gi/o family proteins, with Go more efficient than Gi [515], while N- and P/Q-type VDCCs can be stimulated by Gs proteins. Physiological responses induced by melatonin-mediated inhibition of Ca^{2+} influx or uptake have been found in many mammalian tissues. For example, melatonin inhibits dopamine release from the rat hypothalamus [516], inhibition associated with suppression of calcium uptake by the stimulated tissue and documented at different times of the day [516-518] and causes vasorelaxation in rabbit basilar arteries [519]. It is known that Go and Gi proteins are involved in the inhibition of N-, L-, and P/Qtype VDCCs. Moreover, Gβγ subunits have been suggested to inhibit N-type channels by a PLC-ß stimulated PKC pathway [520]. Thus, it might be expected that stimulation of MTs may lead to the inhibition of N-, L-, and P/Q-type VDCCs via Gαi/o- and Gβγ-mediated pathways. On the other hand, there is growing evidence that melatonin is able to facilitate opening of L-type channels to induce vasoconstriction in the rat [521] and increase the contractility of chick myocytes [522]. The induction of Ca^{2+} influx by melatonin has been reported also in hamster retina [523].

Melatonin may regulate also the SMOCs: these channels are activated by intracellular inositol phosphates [524], so it is possible that melatonin induces the opening of SMOCs via a PLC-dependent pathway. For example, in human prostate cells the increase by melatonin of cGMP leads to enhanced calcium uptake through cyclic nucleotide gated channels [525].

1.4.3.7 Guanylyl cyclase (GC) and cGMP-phosphodiesterase (cGMP-PDE)

It is widely demonstrated that melatonin has an inhibitory function of cGMP production: in the cerebral cortex of the chick, rat pituitary, human prostate epithelial cells and murine melanoma cells [457, 526-528], resulting in cGMP circadian rhythm. This effect is mediated via MT2 and not MT1 [471, 485, 529].

cGMP is an important regulator of SCN circadian rhythms [530] and retina, however melatonin role in regulating cGMP are still not yet apparent. In the retina the activation of the photopigments rhodopsin and opsin by rods and cones activate the rod-specific Gt1

and cone-specific Gt2 G proteins that lead to the metabolism of cGMP in 5'GMP by cGMP-PDE [531]; it is not known whether MTs can utilize $G\alpha_t$ proteins to directly affect cGMP levels or whether they employ $G\alpha_i$ family proteins to modulate pre-stimulated cGMP levels. Alternatively, melatonin may inhibit GC activity by inhibiting the activity of NO synthase (NOS), which produces NO, a known activator of GC [532-533]. The mechanism of melatonin inhibition of NOS activity has not been extensively studied but one hypothesis may be that melatonin block the Ca^{2+}/calmodulin activation of NOS [534].

Melatonin is also able to induce cGMP accumulation in tissues such as human lymphocytes [535], chick pineal gland [536] and in rat anterior pituitary, thyroid, liver and small intestine [537]. These findings suggest that cGMP may be involved in melatonin signalling pathway. The mechanism how melatonin increases cGMP levels are still not clear but may involve the inhibition of cGMP-PDE activity [528] as well as the activation of GC [536].

1.4.3.8 MAPK

Melatonin was shown to be able to activate several MAPK pathways, such as the extracellular signal-regulated kinases (ERKs) and the transcription factor Elk-1, that in human granulosa cells may regulate progesterone production, LH receptor, GnRH, GnRH receptor gene expression, ovarian function [538], and the c-Jun NH2-terminal kinases (JNKs, also referred to as the stress activated protein kinases, SAPKs) in COS-7 cells transfected with human MT1 and MT2 that activated through Gs, Gz and G_{16} [480]. MCF-7 breast carcinoma cells JNK and ERK were activated by MT1 receptor [480].

Melatonin has been proven also in this case to have opposing effects in different cell types. In PT cells it was noticed that melatonin inhibit the ability of IGF-1 to activate ERK activity probably through its inhibitory effect on cAMP production [539, 540], the same effect that mediated the inhibition of the uptake of the promoter of mitogenesis via MAPK kinase pathways linoleic acid by rat hepatoma cells [541].

1.4.4 MT1 and MT2 melatonin receptor distribution and function

Melatonin exerts its role mainly through the binding to melatonin receptors MT1 and MT2. These receptors were widely demonstrated to be present in the central nervous system, in many brain areas. Also MT3 is present in the brain, but its levels are higher in other organs such as the kidney and liver [465]. In the brain the effects of melatonin depend on the area where the receptors are present: in the SCN melatonin modulates the entrainment of

circadian rhythms and may induce sleep [542], in the retina melatonin inhibits the stimulation evoked release of dopamine [471], modulates rod phototransduction pathway and photoreceptor functions and help the retina for the adaptation to low light intensities [543] whereas in the hippocampus melatonin contributes to the memory formation and to the excitation and inhibition of neuronal activity [464, 544]. Other functions of melatonin in the brain are the modulation of dopamine synthesis and release and activation of dopamine receptors [545].

1.4.4.1 SCN

MT1 receptor is demonstrated to be expressed in rodent SCN [217] as well as in human SCN [542].

As explained above, the SCN takes place in the synthesis and release of melatonin from the pineal gland [423, 546]; however, melatonin feedbacks in the SCN and regulates neuronal activity and circadian rhythms [211].

Models to study melatonin influences on the SCN are cellular (mammalian SCN2.2 that express both MT1 and MT2 [448, 488, 529, 547]) and animals (e.i. rodents, whose SCN was reported to express MT1 and MT2 proteins with 37 KDa and the glycosylated form) [448].

One of the classical effects of melatonin was studied in SCN2.2 cells, where melatonin inhibits the forskolin-stimulated cAMP accumulation [448]. A similar effect was observed in mouse SCN slices, where melatonin inhibits the PACAP–mediated cAMP response element binding protein (CREB) phosphorylation, an effect mediated via MT1 [472, 548]. From the retinohypothalamic tract two kinds of synapses are connected to the rodent SCN: glutamatergic, that induces through n-methyl-D-aspartic acid (NMDA) activation Ca^{2+} influx and PACAP, that through PACAP-R1 increases cAMP production [549-551]. Both pathways phopshorylate CREB. However, PACAP is more sensitive during late daytime, signalling darkness, whereas glutamate is more sensitive during early night time, signalling light. Melatonin, thorugh its inhibition of PKA pathway, is able to counteract PACAP signalling [552], but not glutamate signalling [553] and probably this may preserve the responsiveness of the SCN to light-induced phase shifts at night. This mechanism is mediated via MT1 receptor at low melatonin concentrations, but at higher melatonin levels it is also mediated via MT2 [472].

The activation of MT2 in rat SCN and cellular model stimulates PKC activity [448, 488].

Melatonin has an effect also in inhibiting neuronal firing rate in SCN slices especially at dusk [554] through MT1 activation [507, 555]; this effect is due to an increase in potassium conductance and subsequent neuronal hyperpolarization [484] through activation of the inward rectifier potassium channel Kir3 [556]. This function of melatonin on alteration of clock excitability as it shifts from day to night may promote sleep by inhibition of neuronal activity in the SCN and/or other areas of the limbic system.

Melatonin has a function also in phase advancing circadian rhythms at dusk (fall phase of melatonin production) and dawn (rise phase of melatonin production) through activation of MT2 and PKC [487-488, 529, 557]. Interestingly, if physiological concentrations of melatonin (between 30 and 300 pM) are applied on rat SCN slices or SCN2.2 cells for 8 hours during the night the phase shifting and PKC stimulation effects of melatonin are abolished by desensitization of MT2 but not MT1 that was fully recovered 8 hours after 8 hours from melatonin administration [558-559].

1.4.4.2 Pituitary gland (PT)

Melatonin is critical for the regulation of seasonal changes in various aspects of physiology and neuroendocrine function [560-561]. These actions of melatonin are processed in nuclei of the hypothalamus [562] and in the PT of the pituitary [563-564].

In PT melatonin activates Gi and Go that leads to the inhibition of forskolin-stimulated cAMP accumulation [496, 526, 565-566] inducing the inhibition of CREB phopshorylation [472, 497, 552-553]. This inhibition influences the expression of the transcriptional inhibitor inducible cyclic AMP early repressor (ICER) that is rhythmically expressed in the PT.

Another implication of melatonin inhibition of cAMP in rodent PT is the *Per1* expression [567], whose production is repressed by the inhibition of cAMP induced by melatonin [473]. However, the repression of AC after several hours super-sensitises the enzyme by activators [568], leading to higher *Per1* level expression than occur in the absence of melatonin [567]. The temporal regulation of the *Per1* gene by melatonin is thought to contribute to the release of pituitary hormones, such as PRL [569], that regulate circadian rhythms [567].

In mouse strains melatonin-deficient, such as C57BL mice, *mper1* mRNA and mPER1 and ICER protein are completely absent from the PT [564, 567]. If MT1 melatonin receptor-deficient mice are bred with the melatonin-producing C3H genetic background, the offsprings show lower levels of mPER1 protein as compared to their wild-type littermates [567].

However, no MTs seem expressed in human PT [570].

1.4.4.3 Retina

Melatonin receptors MT1 and MT2 are localized in several structures of birds, rats and humans eyes, such as cornea, choroid, sclera, horizontal, amacrine, and ganglion cell bodies as well as the inner segments of rod photoreceptor cells, retinal pigment epithelium (RPE) and retinal blood vessels [543, 571-573]. The wide-ranging distribution of MT1 receptors is indicative of melatonin indirect action on vascular smooth muscle [574] the presence of MT2 receptors in the apical microvilli of cell membrane, but not in the basal membrane of the RPE, suggests that melatonin is involved in photoreceptor outer segment disk shedding and phagocytosis [572]

MT3 is also found expressed in the eyes of rabbits, probably regulating intraocular pressure [575]

The presence of MTs highlight the role of melatonin in the eyes, to regulate rod phototransduction pathway [543] differential regulation of the growth and remodelling of the fibrous and cartilaginous scleral layers which in turn affect eye size and refraction [573] and in the regulation of humor secretion and maintenance of the circadian variations of intraocular pressure [576]. Moreover, melatonin was demonstrated to have a role in physiological adaptation to low light intensities: in mammalian retina rod photoreceptors make synapsis with AII amacrine cells, whose gap junctions modulation by ambient illumination forms the basis for visual acuity. Retinal dopamine inhibits retinal melatonin synthesis [577-579] through $D_{2/4}$ receptors; melatonin inhibits dopamine synthesis [579-580].

Dopamine uncouples homologous AII junctions acting through D_1 receptor [581] whereas melatonin blocks D_1 induced accumulation of cAMP via MT_1 receptors present in AII cells [582]. This inhibition of the light-induced release of dopamine is probably due to the enhancement of GABA activity [583].

1.4.4.4 Hippocampus

Melatonin receptors MT1 and MT2 were found in the dentate gyrus, CA3, CA1 and in the subiculum of the rat hippocampus [584]; however in mice melatonin receptors show a different localization profile, since the MT1 receptor is predominantly expressed in neurons from the CA1 subfield, which is the main output of the hippocampus, whereas the MT2 receptor is especially detectable in the CA4 and CA3 pyramidal neurons, which receive glutamatergic excitatory inputs from the entorhinal cortex [585].

In the rat CA1 melatonin enhances the neuron firing rate [584] and inhibits the long-term potentiation (LTP) in murine hippocampus slices [586] through MT2 dependent pathway, that involves PKA inhibition [586]. The same conclusions about the melatonin-dependent inhibition of LTP were found *in vivo*, by the observation that the processes of learning/memory are impaired in MT2 knockout mice [587]. Probably the excitatory/inhibitory actions of melatonin on the hippocampus are influenced by circadian rhythms of melatonin secretion.

In a human study melatonin was shown to enhance the activation of the left parahippocampus in an autobiographic memory task [588]. The activity of this area of the hippocampus shows diurnal variation, with an enhancement of the activity in the evening [589]. Since the exogenous administration of melatonin could increase the activity only when the endogenous melatonin levels were already increasing, this may indicate an effect of melatonin and circadian rhythm in human memory processing during the night [589] such as memory consolidation [590].

In elder and AD subjects, increasing levels of MT1 and decreasing levels of MT2 in hippocampal CA1-4 subfields were observed [464, 544]. MT1 level changes are probably a compensatory mechanism due to a decreased melatonin secretion, whereas the MT2 level changes were hypothesised to be related to the memory impairment of the diseases. Indeed, administration of melatonin disrupted the inhibitory neuronal functions by reducing the $GABA_A$ receptor mediated currents in CA1 pyramidal cells through activation of MT2 receptors [584] so probably the loss of hippocampal MT2 receptors could disturb the activity of GABA-ergic transmission which may underlie the pathogenesis of AD. Another pathology that melatonin may affect through the influences on GABA transmission is epilepsy. In children, pharmacological doses of melatonin lead to a decrease in the severity and/or frequency of epileptiform activity [591] and pinealectomy in rats increases the probability to develop the processes that follow the long-lasting status epilepticus, including increased neuronal loss with a higher number of spontaneous seizures occurring during the chronic phase [592].

While melatonin does not interact with $GABA_A$ receptors, it is interesting to note that it potentiates the hypnotic effects of benzodiazepine and non-benzodiazepine drugs that enhance $GABA_A$ receptors [593-595]. Co-administration of melatonin during the withdrawal period has been shown to facilitate discontinuation of hypnotic drugs [596-597].

1.4.4.5 Other brain areas

In the murine substantia nigra, ventral tegmental area, nucleus accumbens, caudate-putamen, and in the human prefrontal cortex, putamen, caudate nucleus, nucleus accumbens, substantia nigra, and amygdala MT1 expression was shown [545]

The dopaminergic system is important for movement and psychological factors, such as reward, as well as for producing the reinforcing actions of drugs of abuse, like cocaine. For example, it was shown that melatonin cotreatment can block the development of cocaine-induced behavioral sensitizations, such as anxiety during cocaine withdrawal [598].

In distinct human cerebellar cortex cells MT1 and MT2 are expressed [599]. MT1, for instance, was localized in cell bodies of granule cells and basket-stellate cells. Moreover, MT receptors may have an inhibitory effect by colocalization with the excitatory glutamatergic synapses, leading the hypothesis that melatonin has a role in the posture control and balance [600].

1.4.4.6 Skin

Most dominantly MT1 is expressed in the human skin [601], including kerotinocytes, melanocytes (epidermal and hair follicle), fibroblasts and squamous cell carcinoma and melanoma cells. The MT2 receptor was also detected, but transcripts were present only in neonatal keratinocytes and in one melanoma cell line. Even if melatonin was shown to inhibit cell proliferation in cutaneous melanoma cells and in normal and malignant uveal melanocytes [602-603] it has no effects on the proliferation of dermal fibroblasts grown in medium containing growth factors [601]. In addition to its receptor mediated effects, through its antioxidative mechanism melatonin, has protective effects on UVB-induced damage in dermal fibroblasts and epidermal keratinocytes [604].

1.4.4.7 Gastrointestinal tracts

MTs are expressed in several tissues of the gastrointestinal tract of rodents and humans: MT1 is expressed in liver, kidney [605] and gallbladder [606], MT2 is expressed in lung and duodenum [450] and MT3 is expressed in liver, kidney, lung, muscles, intestine [465].

Because of its local synthesis by enterochromaffin cells [607] the gastrointestinal tract and the gallbladder contain high concentrations of melatonin [608]. However, the function of melatonin in the gallbladder is only speculative. It is known that melatonin can modulate gastrointestinal ion transport processes and motility [417]. Furthermore, in rats, melatonin alters cholecystokinin-induced changes of ileal motility [609]. Melatonin may also interfere with NO signaling [533], possibly influencing gallbladder contraction.

In the duodenum melatonin influences HCO_3^- release from the duodenal mucosa [610-611] with a mechanism under nervous control [612]: melatonin activates the release of the intracellular calcium in the enterocytes which, in turn, activates apical electroneutral HCO_3^- /Cl^- exchange [613].

Many pancreatic cell models, such as pancreatic islets and the insulinoma pancreatic β cells INS-1, express MT1 but not MT2 [614-616]. Acute melatonin treatments inhibit forskolin-stimulated and glucagon-like peptide insulin secretion through activation of G_i/G_o pathway and insulin promoter activity and CRE–mediated gene expression [614-616]; however, melatonin alone has no effects on insulin secretion. On the contrary, chronic melatonin treatments increased basal levels of insulin secretion and potentiated GLP-1- and forskolin-induced insulin and cAMP production [614]. Regulation of the circadian release of insulin and sensitization of the cAMP system by endogenous melatonin may provide a mechanism by which high morning levels of insulin facilitates glucose disposal following nutritional stimuli [614].

1.4.4.8 Adipose tissue

All melatonin receptors MT1, MT2 and MT3 were shown to be expressed in adipocytes [450, 465, 617].

Melatonin is known to play a role in energy expenditure and body mass regulation in mammals: melatonin can modulate fat mass activation in several species [618-620]. Moreover, classical side effects of the misalignement of circadian rhythm and melatonin pattern in human are oversleeping, carbohydrate craving and overall weight gain [621].

In a cellular model of immortalized adipose tissue MT1 and MT2 were indentified to be expressed.

Long melatonin treatment leads to a decline in cAMP accumulation and inhibition of cGMP levels, probably mediated by MT2, that produces a decrease in the insulin-mediated glucose transport GLUT4 and glucose levels [485].

Inguinal and epididymal rat adipocytes express MT1 and MT2; in inguinal adipocyets melatonin inhibits isopropanol-stimulated lypolysis via Gi/Go pathway [622], whereas in epididymal adipocytes melatonin in the presence of insulin increases leptin secretion and counteracts forskolin-induced inhibition of leptin secretion and mRNA expression. However, insulin or melatonin alone did not modify leptin secretion suggesting cross talk between these hormones to modulate leptin production from adipocytes via MT1 receptor activation [623].

1.4.4.9 Reproductive system and seasonality

Several tissues in the reproductive system express MT: MT1 was shown to be expressed in breast [624] testis [625] ovary [626] and MT2 is expressed in myometrium and granulosa cells [450].

Some evidences that may connect melatonin with the uterus functions are a circadian rhythm of uterine contractility and electrical activities [627] and the evidence that birth rate occurs especially during the night [628]. During rat pregnancy oxytocin receptors are upregulated and MT receptors are downregulated, probably due to the inhibitory effect of melatonin alone on uterus contraction [629]. However, in the presence of noradrenalin, melatonin can augment contractile forces in human myometrial strips [630].

Administration of exogenous melatonin to women induces a decrease in luteinizing hormone (LH) secretion, blocks ovulation, and the luteal phase, increase in progesterone, without affecting follicle-stimulating hormone (FSH) or inhibiting estradiol [631] Acute suppression of LH levels was also observed in men with a negative correlation between inhibin and the LH/testosteron ratio [632]. These effects are thought to be mediated by regulating hypothalamic gonadotropin release [633]. However, recent studies suggest that melatonin can also exert effects on this axis by directly binding to granulosa cells in the ovary [634]. A study in human granulosa cells showed that both types of melatonin-receptors are present and that melatonin can upregulate LH mRNA receptor [538]. LH is essential for the initiation of luteinization. Furthermore, melatonin treatment enhanced the hCG stimulated progesteron secretion. An additional important result of this study was an inhibition of gonadotrophin-releasing hormone (GnRH) and GnRH-receptor expression. GnRH in the ovary is suggested to be an important paracrine and/or autocrine regulator and may be involved in the regression of corpus luteum [635]. Therefore these results may point to a possible involvement of melatonin in the maintenance of the corpus luteum during pregnancy.

Endogenous released melatonin resulting from changes in day length modulates reproduction in seasonal breeders [561, 636-637]. This effect is mediated by modulation of the hypothalamic–pituitary–gonadal (HPG) axis function through activation of MTs in hypothalamic GnRH releasing neurons, PT of the anterior pituitary, gonadotrophs and lactotrophs of pars distalis, as well as testes and ovaries [625, 638-640].

In laboratory rodents the effect of melatonin on the HPG axis is predominantly inhibitory. In immortalized GnRH releasing cells melatonin decreased the expression of GnRH mRNA in a 24 hours cyclical manner through MT1 and MT2 signalling [638]. GnRH in turn controls

the secretion of the gonadotrophins, LH and FSH that regulate reproductive functions at the level of the gonads.

Activation of MTs expressed in neonatal rat pituitary gland inhibits GnRH-induced LH release, cAMP and cGMP accumulation and the increase in intracellular Ca^{2+} through activation of a pertussis toxin–sensitive G protein–coupled receptor [641-643]. The mechanism(s) by which melatonin modulates gonadotrophin secretion from the pituitary gland is complex and appears to involve primarily activation of MT1 melatonin receptors [631, 644]; however, participation of MT2 receptors[645] cannot be excluded.

In hamster testicular Leydig cells, melatonin inhibits basal and chorionic gonadotropin-stimulated cAMP and androgen (testosterone and androstene 3α-diol 17β diol) production through activation of MT1 receptors [625]. In this animal model melatonin increases local corticotrophin-releasing hormone and downregulates the expression of steroidogenic acute regulatory protein and other key steroidogenic enzymes [625].

1.4.4.10 Cardiovascular system

A link between melatonin function and cardiovascular functionality was highlighted by the observation that patients with coronary heart disease and non-dipper hypertensive patients (patients who do not exhibit a normal decline in blood pressure at night) have significantly lower nocturnal melatonin secretion than healthy controls [646-649].

In the cardiovascular system the three main MTs are found. MT1 is present in the peripheral and cerebral arteries [650-651] as well as MT2 [450]; MT2 is also present in the aorta [450] and in the heart are present both MT2 [450] and MT3 [465]. Expression of MT1 receptors in human coronary arteries exhibits 24 hours variation with lowest values detected after 02:00 am up to the late morning hours and a progressive increase seen after 01:00 pm until midnight [651]. The expression of melatonin MT1 receptor in the coronary arteries followed a similar rhythm and amplitude in coronary patients and in controls [651]. In contrast, melatonin MT2 receptor expression in coronary arteries, aorta and left ventricles of patients with coronary heart disease differed from that of controls [652].

Although melatonin receptors MT1 and MT2 were found in human left ventricular tissue [650, 652] melatonin role in left ventricular function has not been fully clarified. Melatonin stimulates high voltage activated calcium currents in chick embryonic heart tissues [522] and in isolated rat papillary muscle it possesses anti-adrenergic effect inducing reduction

of contractility increase [653]. Furthermore, melatonin can inhibit isoproterenol-stimulated cAMP production in primary heart cell cultures [654].

Better known is melatonin role in vessels: MTs mediate both vasoconstriction and vasodilatation through activation of distint MTs, probably by modulating NO and noradrenergic effects.

In porcine coronary arteries melatonin induced vasoconstriction via inhibition of NO effects and potentiation of serotonin effects [655]. In rat caudal artery melatonin potentiates the adrenergic nerve stimulation and norepinephrine contraction without affecting the vascular tone [656]. In this case vasoconstriction is probably mediated by decreases in cAMP-dependent phosphorylation of calcium-activated potassium channels (BKCa) through G_i/G_o protein-coupled MT1 melatonin receptors present in the smooth muscle, although participation of receptors localized in the endothelium cannot be ruled out [483, 657-658].

Also the vasoconstriction of the cerebral arteries is mediated via G_i/G_o and BKCa channels [659-660]. In this way MT1 melatonin receptor may have a role in the regulation of cerebral blood flow [661]. It is conceivable that melatonin-mediated vasoconstriction by modulating vascular tone may attenuate diurnal fluctuations in blood pressure keeping cerebral flow constant [662].

From rat studies on arteries, melatonin seems to mediate vasodilatation through MT2 receptor [657, 663]. Moreover, vasodilation and increase in blood flow induced by melatonin in distal skin regions may underlie the concomitant heat loss and hypothermic effect of this hormone [664]. Melatonin is involved in the control of intraocular pressure during the daily photoperiod probably through MT3 receptor [575].

In bigger arteries such as rat and rabbit aorta melatonin was shown to mediate vasodilatation [665] through the elevation of NO, potentiation of acetylcholine- and/or inhibition of noradrenaline effects [666].

In humans, treatment with melatonin is effective for lowering blood pressure in normal men [667] and postmenopausal women [668], in the hypertensive type 1 diabetic adolescents [669] and in patients with nocturnal hypertension [670] without affecting the heart rate.

Melatonin effects on thermoregulation are also important in humans: the administration of melatonin leads to vasodilatation in distal body regions reducing CBT and inducting sleepiness [671-672]. Exactly which MTs are involved in these effects remains to be investigated. These studies confirmed the hypothesis that melatonin in humans is involved in the circadian variation of body temperature and induction of sleep.

1.4.4.11 Immune system and cancer

Melatonin regulates hematopoiesis, through its action on specific receptors on bone marrow cells [673] and has a function in the physiology of the immune system. The first strong evidence of a connection between melatonin and the immune system comes from the observation that pinealectomy caused thymic involution and suppressed immunity [674] and that inhibition of melatonin synthesis suppressed both cellular and humoral responses in mice [675].

Melatonin is synthesized by human lymphocytes and the evidence that human lymphoid cells and mice immune system express MTs (respectively MT1 and RZR/RORα [676] and MT1 and MT2) [677] suggests an autocrine and/or paracrine role of melatonin in regulating immune responses [419, 678-679] participating in the production of IL-2 and IL-2 receptor [419]. Also in other lymphoid organs, such as the thymus and spleen of mice, a significant expression of membrane MT1 and MT2 receptors and of nuclear RZR/ROR α, occur [677].

Melatonin is an immunomodulator: indeed it stimulates the immune system [678] and decreases the acute inflammatory processes. In seasonal animals short day duration, the period of maximal melatonin secretion, corresponds to enhancement of the immune function [680]; diurnal rhythms of experimental granulomatous inflammation in rodents are blocked by pinealectomy and are re-established by nocturnal replacement of melatonin [681] and an effect of melatonin as enhancement of mice splenic lymphocyte proliferation was measured either during the day or night probably through melatonin receptor MT2 [682-683].

Melatonin reduces acute inflammation in rats by inhibiting leukocyte rolling in the microvasculature through activation of the MT2 melatonin receptor and leukotriene B4-induced leukocyte adhesion to endothelial cells through a MT with the pharmacological characteristics of the MT3 site [684].

One important function of melatonin is mediated through modulating of the production/secretion of cytokinese [685]: melatonin enhances human lymphocytes IL-2 production and monocytes IL-6 production [686] probably mainly through a RZR/ROR α receptors dependent mechanism [687]. In human lymphocytes melatonin counteracts the inhibitory effect of PG-2 on IL-2 production through its MT1 membrane receptor, but nuclear receptor cannot be ruled out [446, 688]. Furthermore, melatonin activates human monocytes by inducing cellular cytotoxic agents, such as interleukins and TNF-α [674]. In clinical trials carried out in metastatic cancer patients, melatonin and IL-1 were found to increase the cluster of differentiation (CD)4/CD8 ratio [680]

The activation of lymphocytes and monocytes/macrophages by melatonin is one of the principal mechanisms by which melatonin prevents tumor development [689-690]. In many studies the co-therapy with IL-2 and melatonin improved the treatment of several tumors, such as advanced tumors of the digestive tract [691].

In addition to immunostimulation, the melatonin/IL-2 relationship may be particularly relevant for immune tolerance. $CD4^+$-$CD25^+$ T regulatory cells represent a critical T cell subset that suppresses peripheral autoreactive T cells that escape thymic negative selection [692] and melatonin and IL-2 may affect T-cell tolerance by inhibiting uncontrolled T cell proliferation and autoimmunity [693]. In fact it has been reported that IL-6 plays a crucial role in driving the differentiation of T regulatory cells into IL-17 secreting T-helper (Th) cells which contribute in Th-1 and autoimmune responses [694]. Thus, in the absence of inflammatory stimuli the immunological function of melatonin might be opposite to that exerted during inflammation and/or infection.

Beyond melatonin effects on cytokine secretion, it reveals also an antiproliferative effects that is why it can be considered as an oncostatic agent (for review, see refs. [695-696])

In animals bearing carcinogen-induced tumors, melatonin treatment reduces tumor number and size, increases tumor latency, and lowers tumor incidence. *In vitro* melatonin inhibits tumor cell proliferation through an effect on the cell cycle [697], interaction with sex steroid–responsive pathways [696] and/or perhaps in part via its free-radical-scavenging potential [698]. Membrane MTs appear to be involved in melatonin oncostatic effect [624, 699-701]. The concentration of MTs can vary on the basis of the tumor [606, 702]. Other mechanisms that involve melatonin in the cell proliferation inhibition are: the inhibitory effects on the expression of estrogen-receptor alpha (ERα) and/or inhibition of binding of the E_2-ER complex to the estrogen responsive element on the DNA, the inhibitory effects of melatonin on calmodulin, which can phosphorylate the ERα thus facilitating the binding of estrogen and in a phase II study it was shown that the administration of melatonin and tamoxifen induced an objective tumor regression compared to tamoxifen alone [703].

1.4.4.12 Adrenal gland

Few studies suggest a link between MT activation and adrenal gland effects. In the capuchin monkey MT1 is expressed in the fetal and adult adrenal gland [704-705]. In adult adrenal explants and dispersed cells melatonin inhibited the ACTH–induced cortisol production, corticotropin releasing hormone–induced cortisol secretion and decreased dibutyril cAMP-induced cortisol secretion [704].

Melatonin did not appear to regulate cortisol secretion, although it inhibited ACTH-induced increase in 3β hydroxysteroid dehydrogenase mRNA gene expression in fetal adrenal gland, suggesting a selective effect of melatonin on the cortisol synthesis pathway [705]. Moreover, suppression of melatonin by light treatment in capuchin monkey increased blood cortisol in the newborn; this effect was normalised when mothers were treated with melatonin [705].

1.4.5 Melatonin interactions with the circadian system

Several studies in humans showed that phase shifts of major physiological parameters, such as core temperature, endogenous melatonin onset, and sleep timing occur after melatonin administration. Phase delays are observed after morning administration, whereas phase advances occur after evening administration [169]. Furthermore, timed administration of melatonin facilitates the readjustment after acute phase shifts of the light–dark schedule-such as in jet lag and shift work [706]. Finally melatonin supplementation results in entrainment of free running circadian rhythms in blind people [707]. All of these studies suggest that melatonin, after binding to MT-receptors in the SCN, participates in synchronizing circadian rhythms.

1.4.5.1 Melatonin as zeitgeber in animal mammalian studies

Physiological concentrations of melatonin significantly phase advanced the peak of circadian rhythm of neuronal firing in the SCN brain slice from a melatonin-deficient mouse strain, the C57BL/6 [217, 708-709]. Melatonin administered in rat SCN slices at about CT 10-14 is able to cause 2 or 3 hours of electrical activity peak phase advance, whereas treatment at other times had no apparent effect [710]. The effect on the phase shift of SCN firing rate is mediated by MT2.

Another hint that melatonin has chronobiotic properties comes from cellular studies, where coculture of chick astrocytes with chick pinealocytes or administration of exogenous melatonin cycles could entrain metabolic rhythms of 2-deoxy ^{14}C-glucose (2DG) uptake and/or clock gene expression in cultured astrocytes [711].

Most of the original chronobiotic effects of melatonin were observed in photoperiodic seasonal breeders such as hamsters and sheep. Suitably timed treatment over periods of weeks was equipotent with artificial changes in daylength in the induction or suppression of seasonal events such as reproductive competence. These major physiological changes were accompanied by clear changes in the circadian waveform of PRL secretion and in

the rhythmic characteristics of LH and FSH secretion, suggesting effects of melatonin on central rhythm generator(s).

In mammals pinealectomy has no a dramatic effect on the locomotor activity, suggesting a redundancy of this circadian output [712-713]. However melatonin may play an important role in circadian organization: after pinealectomy subtle desynchrony of several physiological functions has been described [714] and the reentrainment of rat locomotor activity rhythm is modified after a phase-shift of the light/dark (LD) cycle. One week after pinealectomy the firing rate rhythm of SCN neurons *in vitro* is altered, as well as the daily rhythm of responsiveness to melatonin [715]. Melatonin is also known to interfere with the metabolic activity (glucose utilization and protein synthesis) of the SCN [716] and in the rat PT, the MT circadian rhythm density is suppressed after pinealectomy [717].

The exogenous administration of melatonin has important effects on mammalian circadian rhythms, since melatonin can entrain free-running activity in rodents, if the administration is given at the activity onset, although activity arousal per se has a phase advancing effect of the locomotor activity [718]. Melatonin administrered in drinking wate, or canulated chronic infusion up to 16 hours, was able to entrain Syrian hamsters [719-720]. In chronical parfused animals the phase angle difference between the entrained rhythm and the zeitgeber (melatonin) depended upon the duration of the infusion period [720]. In Syrian hamster the daily infusion for more hours of a lower melatonin dose is more effective in entraining than a higher melatonin dose for just one hour; however, the majority of animals showed entrainment, whereas another part showed transient entrainment and few animals didn’t entrain to melatonin administration [721].

When free running animals were entrained with melatonin at different day duration (melatonin treatment once a day, with variable day length), a good entrainment was obtained only when melatonin administration was given in a stepwise manner; this experiment also shows that in rats the limit phase advance value of melatonin entrainment is around 35 minutes and entrainment occurred at about CT 12 [722].

Infusion of Mel has been reported to entrain hamsters or *Arvicanthis ansorgei*, a diurnal rodent, by inducing phase advances when the free-running period was longer than 24 hours and phase delays when the period was shorter than 24 hours [723]. All these observations strongly suggest that the effects of exogenous melatonin are dependent on the period before entrainment.

In rats, when submitted to a 5 hours phase advance of the dark onset in LD conditions and injected daily at the new dark onset, they reentrained with a decreased latency, although

some of the animals phase delayed, whereas others phase advanced [718]. Moreover, a locomotor activity phase shift effect of melatonin was obtained when the administration was at dusk (CT 10) but not at dawn (CT 22–CT2). These results suggest that nocturnally released melatonin may functionally desensitize MT2 melatonin receptors in the SCN and preclude phase shifts at dawn [724].

Also in mice melatonin administered at CT 10 phase advanced the onset of circadian activity rhythms *in vivo* and accelerated the rate of re-entrainment of wheel-running activity rhythms after an abrupt advance of dark onset, through both MT1 and MT2 function [529, 557, 709].

However, since to obtain entrainment the doses of melatonin have to be high, leading to a 100 to 1000 fold higher serum levels than the endogenous melatonin at nighttime suggest that the chronobiotic effect of Mel appears to be pharmacological rather than physiological [716, 719-720].

1.4.5.2 Melatonin as zeitgeber in human studies

Melatonin was shown in several studies to entrain human circadian rhythms. Most of the studies were performed to ameliorate the free-running condition of totally blind individuals. The majority of these subjects could benefit from melatonin treatment [117, 169, 725-726]. The major parameter that is measured in these experiments is the sleep-wake cycle, whose entrainment is the major cause of problems of this kind of people. Indeed, melatonin could stabilize the sleep-wake cycle, probably through its pro-hypnotic effects. Indeed, melatonin is reported to have a role in sleep initiation as the trigger for opening the circadian “sleep gate”, acting as a sleep regulator [59, 727-731]. The pro-hypnotic effects of melatonin are more evident during daytime, when melatonin levels are low: it lowers body temperature and induces fatigue while concomitantly producing a brain activation pattern resembling that which occurs during sleep [588-590]. When melatonin (at doses ranging from 0.5 mg to 5 mg) was administered to blind people once daily at the normal bedtime hour, it entrains free running circadian rhythms of most blind subjects and improves nocturnal sleep and daytime alertness [169, 732-733].

Melatonin (0.5 mg/day) could entrain DLMO or cortisol rhythms of blind subjects only when it was administered in the phase advancing portion of the curve [169, 725], but also when other circadian parameters are not entrained, the sleep-wake cycle is more stable [726].

In a very well controlled study on sighted subjects in dim light condition for several days melatonin entrained the rhythms or at least shortens the CBT period of most of the

subjects immediately treated with 5 mg of melatonin whereas if melatonin was administered after few days of free-running condition its effects where broader, inducing both phase advancing in most subjects, phase delay or entrainment of CBT in few subjects, whereas another minor part continued to free-run. These results indicate that melatonin was unable to synchronize core temperature consistently, even if in the majority of subjects the sleep-wake cycle could be synchronized [261].

A reason that may explain the failure of melatonin to entrain may be associated with the intrinsic period length of the subjects themselves. A longer period needs a greater daily phase shift to maintain entrainment and in some cases this may exceed melatonin phase shifting ability (this of course also applies to sighted subjects, suggesting that late chronotypes need greater phase shifts than early chronotypes to remain entrained).

Sleep-wake cycle of aged people becomes more stable after melatonin administration when subjects are treated from 0.5 to 2 hours before bedtime [167, 734-738].

1.4.5.3 Melatonin PRC

Melatonin was demonstrated to have chronobiotic properties; indeed it is able to phase shift human circadian rhythms. Many PRC experiments attempted to understand the correlation between time of melatonin treatment and shift of circadian rhythms [166, 739-740]. In these experiments melatonin was administered to sighted subjects at the same time of the day for few days and DLMO was chosen as circadian marker to determine shifting properties of melatonin. In these studies melatonin was demonstrated to phase advance sleep, CBT and the endogenous melatonin rhythm when administered at CT of 6 - 12 hours (with a peak around CT 9), that corresponds to about 6 hours before and 4 hours after the initial onset of endogenous secretion, while melatonin phase delays the clock when administered during CTs around 18-24 hours with a peak around 20 hours [166, 739]. Melatonin PRC magnitude and the direction of the shift is different compared to the light PRC: light has about the double of the magnitude of shift compared to melatonin shift, whith a phase advance maximum around CT 6-12 hours and a phase delay maximum around CT 0-6 hours [263, 739]. Moreover, bright light was demonstrated to have a type 0 PRC, whereas melatonin has a type 1 PRC.

Although the PRCs for melatonin have provided to date the template to determine the time of melatonin administration needed for an appropriate phase shift (advance or delay), they do have some methodological problems. A classic PRC assesses phase shifts following a single pulse of the zeitgeber under free-running conditions. The use of repeated doses of

melatonin, sighted subjects in entrained conditions, possible light confounds and transience following the melatonin dose renders not ideal the current PRCs for melatonin. Indeed, in more environmental controlled conditions melatonin oral administration failed to demonstrate a phase delay function of a single melatonin administration in the morning [724], whereas a single melatonin administration in the evening could lead to a significant phase advance [741]. However delays were observed after intravenous infusion of physiological night-time melatonin levels [740]. Probably different results are due to differences in oral melatonin pharmacokinetics between the individuals.

A number of studies have addressed the ability of melatonin to hasten circadian adaptation to simulated phase shift [742]. Shift workers indeed show a misalignement of internal circadian rhythm with the external time, resulting in a multitude of negative symptoms including poor performance and reduced alertness during night work and poor daytime sleep at home. In environmental isolation, studies using suitably timed melatonin treatment (5 mg) and advanced phase shift, rather than delay phase shift, have shown an increase in the rate of re-entrainment of temperature, hormonal, and electrolyte rhythms but with inconsistent effects on sleep [743-744]. Melatonin is able to improve day-time sleep in shift workers, but the alertness during the subsequent night work is unaffected [745-746].

Many other studies demonstrated the efficacy of melatonin to alleviate eastward perceived jet lag (primarily sleep disturbance) [747], but not westward [748], especially if the eastward travel is associated o advancing in bedtime starting few days before the shift till few days after the shift [749].

1.4.5.4 Melatonin and still open questions

As mentioned above, melatonin has multiple roles in many organs; however its function in the circadian system is still not totally understood. The importance of melatonin in chronobiology is documented by many reports in which a decrease in melatonin level is associated to pathologies with strong circadian behaviour impairments such as sleep-wake cycle disturbances (i.e. Alzheimer’s disease, autism but also ageing). Several blind people can benefit from a daily intake of melatonin, to help them to entrain within the 24 hours of day length, but melatonin action seems to be “individual” and effectiveness is not granted. The reasons for non response as well as the right dosage and application time are still under debate.

Moreover, the well conserved circadian presence of melatonin in all biological fluids suggests that the neurohormone has an important function in periphery, but that fact has to be confirmed in further studies.

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2. THE PHYSIOLOGICAL PERIOD LENGTH OF THE HUMAN CIRCADIAN CLOCK IN VIVO IS DIRECTLY PROPORTIONAL TO PERIOD IN HUMAN FIBROBLASTS.

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Running title: Circadian period length in humans

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ABSTRACT

Background: Diurnal behavior in humans is governed by the period length of a circadian clock in the suprachiasmatic nuclei of the brain hypothalamus. Nevertheless, the cell intrinsic mechanism of this clock is present in most cells of the body. We have shown previously that for individuals of extreme chronotype ("larks" and "owls") clock properties measured in human fibroblasts correlated with extreme diurnal behavior.

Methodology/Principal Findings: In this study, we have measured circadian period in human primary fibroblasts taken from normal individuals, and for the first time compared it directly with physiological period measured *in vivo* in the same subjects. Human physiological period length was estimated via the secretion pattern of the hormone melatonin in two different groups of sighted subjects and one group of totally blind subjects, each using different methods. Fibroblast period length was measured via cyclical expression of a lentivirally delivered circadian reporter. Within each group a positive linear correlation was observed between circadian period length in physiology and in fibroblast gene expression. Interestingly, although blind individuals showed on average the same fibroblast clock properties as sighted ones, their physiological periods were significantly longer.

Conclusions/Significance: We conclude that the period of human circadian behaviour is mostly driven by cellular clock properties in normal individuals, and can be approximated by measurement in peripheral cells such as fibroblasts. Based upon differences among sighted and blind subjects, we also speculate that period can be modified by prolonged unusual conditions such as the total light deprivation of blindness.

INTRODUCTION

Nearly all aspects of human daily behavior and physiology are governed by a master clock in the suprachiasmatic nuclei (SCN) of the hypothalamus. This intrinsic oscillator not only governs sleep and wake timing, but also rhythms of temperature, the hormones melatonin and cortisol, mood and cognitive acuity, cardiac, respiratory, and renal function, and most aspects of digestion and detoxification [1]. These rhythms are entrained to 24 hours by the environmental light-dark cycle primarily via a subset of photosensitive retinal ganglion cells that project directly to the SCN [2]. Using multiple hormonal and neuronal signals, the SCN "master" clock in turn entrains peripheral clocks of similar molecular mechanism present in most cells of the body [3].

In humans and other organisms, the timing of 24-hour behavior is governed by the period length of the circadian oscillator. This period is approximately, but not exactly, 24 hours long ("*circa diem*"), and has a reported population range of 23.47 – 24.64 in laboratory conditions [4,5,6,7,8,9,10,11] Short periods lead to behavior occurring at an earlier clock time in some individuals (so-called "larks"), and long periods to later timing of behaviour in others ("owls") [9,12,13,14], at least in young adults. Mutations in clock genes affect period length, and can lead to circadian rhythm sleep disorders such as Advanced Sleep Phase Syndrome (ASPS) [15]. Measurement of circadian period length is therefore a useful step in assessing circadian clock function, either experimentally or clinically.

Determination of the period of circadian behavior in sighted subjects requires prolonged subject observation under controlled-light laboratory conditions. In humans, these studies are expensive and labor-intensive. In one protocol, subjects are kept for multiple days under 'constant routine' conditions consisting of sustained wakefulness or scheduled sleep episodes under continuous dim light with constant posture and frequent isocaloric meals [16,17]. Alternatively, circadian period can be measured under constant dim light for several weeks [18]. Finally, it can be measured under 'forced desynchrony' (FD) conditions where the sleep-wake cycle is scheduled to day-lengths that are outside the range of entrainment for the circadian pacemaker, conditions which force the clock to exhibit its endogenous period. Since it is not ethically possible to keep human subjects in constant routine protocols for prolonged periods, FD protocols are thought to offer greater precision. [4]. A further special case employs totally blind individuals, whose circadian pacemaker cannot be entrained by the environmental light-dark cycle. Although under certain

circumstances these individuals can become entrained to the 24-hour day via nonphotic cues such as exercise, food, and activity [19,20], in other cases they exhibit ‘non-24-hour’ rhythms in melatonin, cortisol or temperature under both laboratory and real-world conditions that provide a direct estimate of their physiological period [21].

Given the intensive experimental control required to assess human physiological period, our laboratories have taken advantage of the duplication of the molecular mechanism of the circadian clock in most mammalian tissues to measure period length in human fibroblasts. To do this, we infect cells with a lentiviral reporter containing the luciferase gene under control of a circadian promoter. Period length in infected cells can then be measured via real-time bioluminescence [22]. In mice with mutations in circadian clock genes, a qualitative correlation has been observed between behavioral period measured by wheel running activity and fibroblast period measured by lentiviral reporter; and in humans, extreme early-type individuals had on average shorter fibroblast periods than extreme late-types [13]. Although these correlations among mutant animals or extreme chronotypes are promising, it remains unclear how well human period length is predicted from measurements in peripheral tissues in ordinary individuals, and whether these values correlate with sleep-wake timing *in vivo.*

In this report, we have for the first time directly compared estimates of human physiological circadian period with the period measured in fibroblasts from the same subjects. We used three different subject groups whose physiological period was estimated via different protocols, and which showed different population averages for physiological period. In each trial, the correlations that we observed suggested that this easy cellular method could be used as a biomarker for human clock properties.

RESULTS

Human fibroblast circadian period *in vitro* correlates with estimates of human physiological period measured *in vivo* in normal individuals.

As described in Methods, we recruited subjects that had participated previously in one of five different studies of physiological period in three countries (Basel, Switzerland; Novosibirsk, Russia; and Guildford, United Kingdom). Four protocols estimated physiological period of sighted subjects under controlled laboratory conditions designed to minimize the effects of environmental light, exercise, food, and sleep upon the workings of the circadian oscillator. Physiological period was determined by the timing of melatonin, a hormone produced at night in circadian fashion and measurable in human saliva. A fifth study focused upon totally blind individuals. For such people, freerunning physiological period has been shown previously to remain independent of the solar day even in a home environment under some circumstances [21]. Hence, subject period could be measured at home over several weeks via the periodicity of the melatonin metabolite 6-sulfatoxymelatonin excreted in urine.

From each subject, two skin biopsies were taken and fibroblasts were cultivated from them. The circadian period length of these fibroblasts was then measured via transduction of a lentiviral circadian reporter and subsequent long-term bioluminescent monitoring. Figure 1B shows a portion of data from fibroblasts of the same subject whose physiological period is shown in Figure 1A.

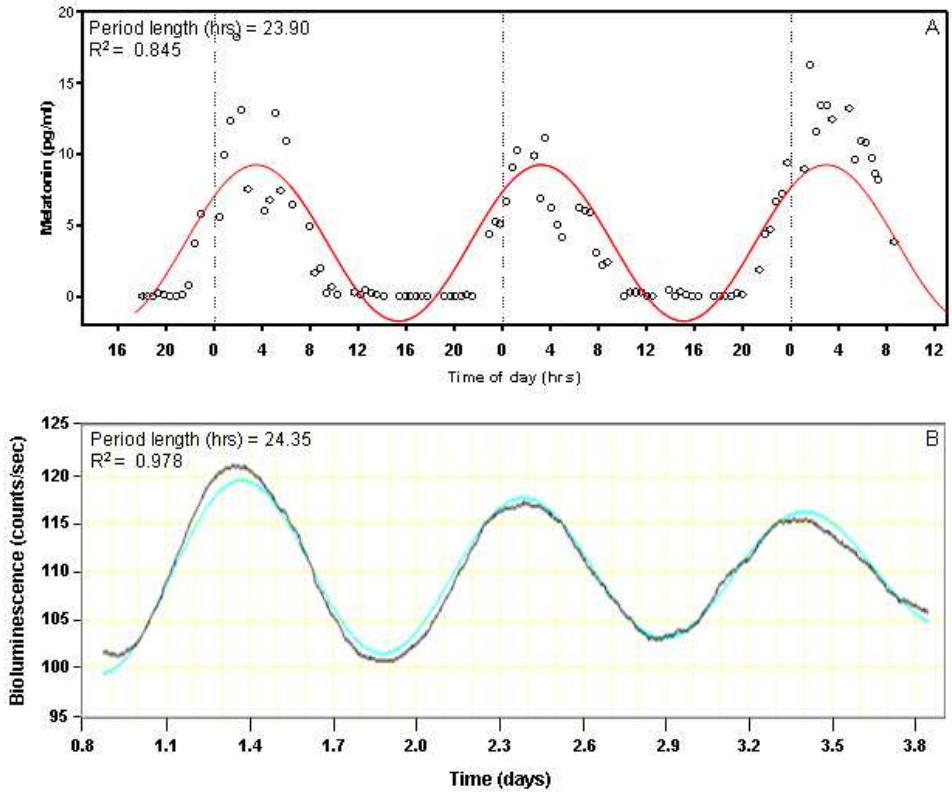

Figure 1. Determination of circadian period *in vivo* and *in vitro.*
A. *In vivo* period length was obtained from salivary melatonin content profiles of normal sighted subjects under a constant routine protocol for three consecutive cycles. Data from one representative subject (Start25) are shown. Open circles, melatonin measurement values. **B.** From the same subject, fibroblast cultures were obtained from two separate biopsies and infected with a lentiviral bioluminescent circadian reporter. After synchronization with dexamethasone, circadian oscillations in bioluminescence were recorded from eight measures over five days. Three cycles of this oscillation are shown aligned with the physiological data of part A. Period calculations for both panels were conducted by cosinor fitting and the best-fit curve is shown in color, along with its period length and goodness of fit R2.

For all subjects, estimates of physiological *in vivo* period were compared to fibroblast period measured via transduction of a lentiviral circadian reporter and subsequent longterm bioluminescent monitoring. The results from 9 subjects measured from two studies in Basel are shown in Figure 2A. Results from a further 11 subjects participating in two studies in Novosibirsk are shown in Figure 2B. Finally, results from 8 totally blind individuals measured in Guildford are shown in Figure 2C. All data are graphed together in Figure 2D. In all groups, positive correlations were observed between fibroblast period and physiological period measured in the same subjects, and Bland-Altman statistics among all subjects show an absence of systematic error between measurements *in vitro* and *in vivo* (Supplementary Figure 1). Table 1 shows the specific values and correlation coefficients obtained from each subject population.

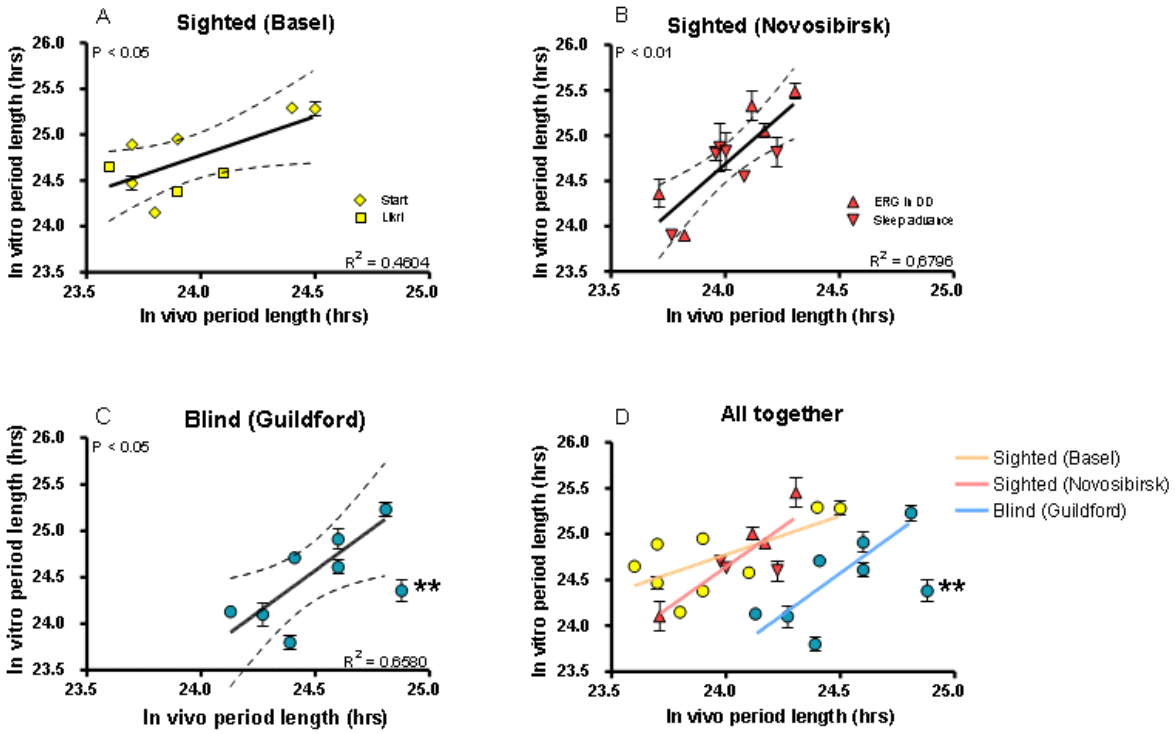

Figure 2. Relationship between physiological period length and fibroblast period length.
A. *In vitro* period length was obtained from skin fibroblasts from nine sighted human subjects of normal chronotype and compared to physiological period in the same individuals measured in two constant routine studies (Basel Start and Likri). The solid line shows the best-fit linear regression (1st polynomial order) represented by the data, and the dashed lines represent 95% confidence intervals to the indicated regression line, with goodness-of-fit R2 shown at lower right. The overall p-value (vs. null-hypothesis slope of 0) is shown at upper-left. For this panel and also panels B and C, identical statistical measures are depicted. In addition, the range and average for each group of subjects are shown in Table 1, and data for individual subjects are listed in Supplementary Table 1. **B.** The same comparison was performed for 11 sighted subjects whose physiological period was measured in controlled laboratory conditions in two studies (Novosibirsk). **C.** A further comparison was performed for 8 totally blind subjects whose physiological period was measured at home (Guildford). Fibroblasts from the asterisked subject showed abnormal clock properties *in vitro* at different temperatures and were excluded from statistical analysis. **D.** The results of all five studies are graphed on the same axes: Yellow, Basel; Red, Novosibirsk; Blue, Guildford.

Table 1. Period lengths *in vivo* and from fibroblasts.

Subject location	N	τ *in vivo*	τ *in vitro*	r^2
Basel	9	24.00 ±0.33 (23.60-24.50)	24.71 ±0.38 (24.15-25.29)	0.46
Novosibirsk	11	24.07 ±0.20 (23.71-24.31)	24.77 ±0.42 (24.10-25.45)	0.68
Guildford	8	24.52 ±0.27 (24.13-24.92)	24.46 ±0.48 (23.80-25.23)	0.66

Table 1. Period lengths obtained from subjects by physiological measurements and from fibroblast molecular analyses.

Blind individuals showed longer physiological period but equivalent fibroblast period.

Average physiological period lengths were significantly longer for the blind subjects than for the sighted subjects (Figure 3A), as reported previously [4,21]. Surprisingly, average fibroblast period was the same for these three populations when measured under identical conditions (Figure 3B). In other words, cellular fibroblast periods were similar among the groups of subjects even though physiological periods varied. Therefore, fibroblast measurements succeeded in capturing inter-individual differences in period among members of each group (whether they were blind or sighted), but were insensitive to apparent differences between blind and sighted individuals.

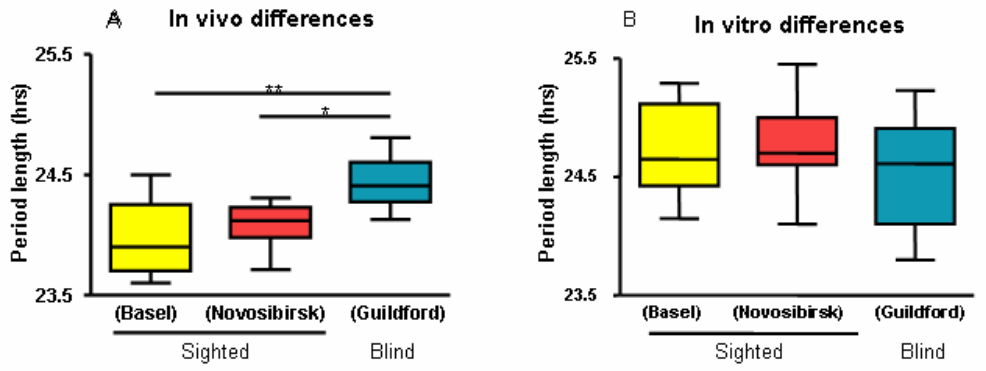

Figure 3. Relationship between population averages of human physiological period length and fibroblast period length.
A. The average *in vivo* period lengths obtained from both protocols with sighted individuals were significantly different from that of blind subjects ($p<0.01$, 0.05). **B.** Nevertheless, the fibroblast-derived values were not significantly different among all three populations. (1-way Anova). Data are presented as a standard boxplot, with bars showing the smallest and largest observed values and box dimensions and midline reflecting lower quartile (Q1), median (Q2) and upper quartile (Q3) values.

DISCUSSION

The fundamental mechanism of the mammalian circadian clock is cell-autonomous, and is based upon feedback loops of transcription and translation that use identical components in most cells of the body [23]. In fact, long-term recordings suggest that the intrinsic clocks in peripheral cells like fibroblasts are probably as robust as those in the “master clock” of the brain SCN [24]. Probably because of these mechanistic similarities, our laboratory has been able to show previously that clock properties of fibroblasts taken from subjects of extreme chronotype (i.e. “larks” and “owls”) generally correlate with the results of questionnaires about subject daily behavior. Many of these extreme subjects, however, showed normal fibroblast period length. We ascribed their behavior to alterations in other clock properties like amplitude [13].

Thus, it remains unclear to what degree the genetically encoded period length of the human circadian clock, as reflected in peripheral tissues like fibroblasts, controls daily behavior in normal individuals whose period lengths vary relatively little. Herein, we have rigorously examined this matter by comparing directly circadian period length measured from human fibroblasts and that estimated physiologically in the same individuals, under different experimental conditions. In the five studies from three laboratories that we have analyzed, we have observed good correlations between period lengths of the circadian oscillator *in vivo* and *in vitro*, even among individuals whose clock properties vary very little (Figure 2).

In vivo, circadian period length under free-running conditions has been shown previously to correlate well with circadian phase under entrained conditions. This circadian phase is best reflected in outputs of the circadian oscillator, such as sleep-wake timing [9,14]. In retrospect, we were interested to know to what extent fibroblast period could predict sleep-wake timing, but our studies were not designed to test this hypothesis and in most cases the rigorous routines of our subjects also controlled sleep-wake. Nevertheless, for the six subjects participating in a constant routine protocol in Basel, physiological circadian phase was measured on the first night of in-patient study via dim-light melatonin onset, and spontaneous wakeup time the next day by EEG. By comparing these two times, we could estimate a “phase angle” of sleep timing with respect to melatonin, which from previous studies should correlate with period [14]. In our case, we could directly compare this phase angle with subject circadian period length measured both *in vitro* and *in vivo*. A trend was

observed between sleep timing and circadian period length in fibroblasts (Figure 4A) and *in vivo* (Figure 4B), but limited subject number prevented it from reaching significance. A larger study would be needed to answer this question more rigorously.

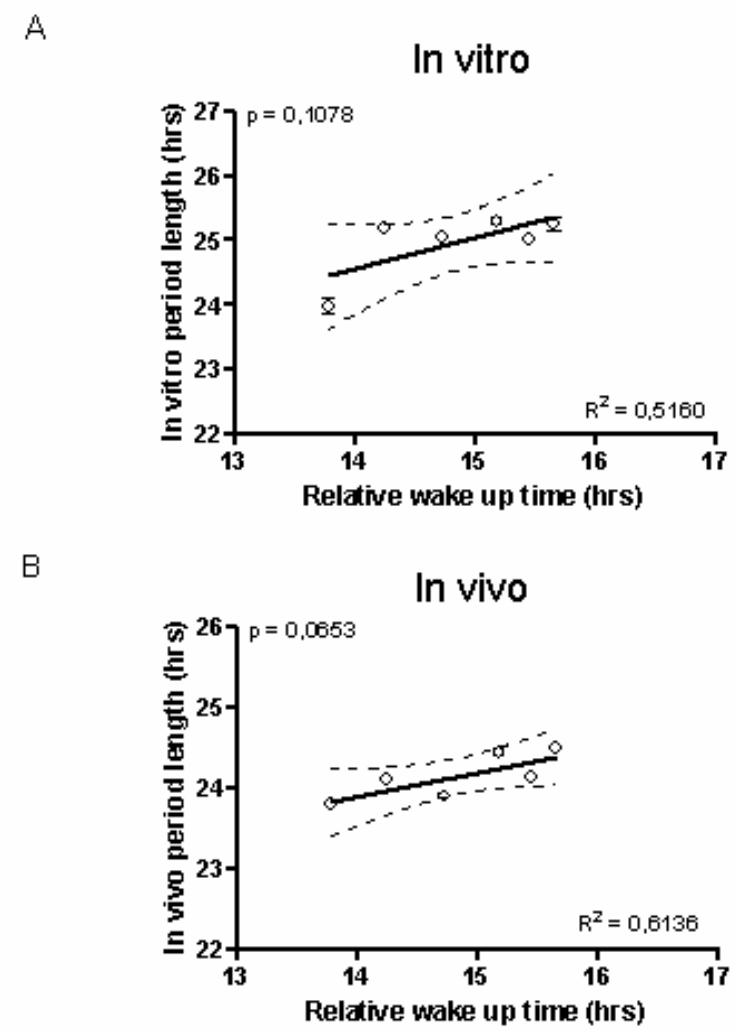

Figure 4. Circadian period and wake timing.
A. Period length measured in fibroblasts is plotted against the circadian phase angle of sleep, calculated as the difference between the measured time of dim-light melatonin onset on the first day of the protocol and the time of subject spontaneous waking that morning. This value could only be determined in one of the five studies presented (Basel constant routine). Linear regression and statistical analyses were as in Figure 2. **B.** Period length *in vivo* is plotted versus the same sleep phase angle.

Approximation of human circadian period by cellular assays could present important advantages. The measurement of human circadian period *in vivo*, either via constant routine or forced desynchrony protocols, is expensive and labor-intensive. An *in-vitro* measure of human circadian period, if validated *in vivo*, could provide an attractive lower-budget alternative. Nevertheless, it should be noted that fibroblast period measures are not a direct proxy for measures of physiological period: for some subjects, considerable differences between the two were observed, and physiological period differences between sighted and blind subjects were absent entirely in fibroblasts.

While it is too soon to be able to draw firm conclusions, it is possible that fibroblast period might prove more useful than physiological period in certain applications. For example,

determination of period length in fibroblasts under carefully controlled conditions might permit separation of genetic effects upon basic clock mechanism from environmental influences present in more complex physiological measurements. Such influences are vividly illustrated by the measurement of sighted and blind subjects included in the present paper. In ours and other published studies of sighted subjects, various protocols have all given period values close to 24 hours [4,6], whereas period length measured in blind subjects at home are longer, and average 24.5 hours [21] (See also Figure 3A,B). This discrepancy is unlikely to result from differences in methodology: recent data illustrates that period assessments in field studies of blind individuals are comparable to period assessment under forced desynchrony in the same subjects (Hull et al., *in press*). In reverse, two groups of sighted subjects in our study were measured in near total darkness (<0.2lux, Novosibirsk), and showed the same shorter period of the sighted subjects that we estimated with constant routine (Basel), or that others have estimated in forced-desynchrony protocols [4].

If the difference is not due to methodology, a basic question arises: are the shorter periods of sighted subjects “aftereffects” of entrainment to light, or is there a fundamental genetic difference between these groups? Our measurements suggest that it is unlikely that this difference is genetic: average fibroblast period was the same in all groups that we investigated, blind and sighted (Figure 3), but still reflected accurately genetic differences causing short and long physiological periods within each group. It is possible, of course, that fibroblast clocks are sensitive to some types of genetic variation but insensitive to others. Nevertheless, in our study we favor the hypothesis that circadian properties between blind and sighted groups differed for physiological reasons rather than genetic ones: for example, human physiological period length might be modified by prior light history and/or retinal function [25]. In support of this idea, individuals recently entrained to a simulated Martian day length showed a longer period length afterward [26].

It should be mentioned that the period of fibroblasts is not invariant, either: their circadian clocks are temperature-overcompensated [27,28]. Thus, whereas normal biochemical reactions slow as temperature decreases, circadian clocks increase in speed. As a result, period length measured from fibroblast cells *in vitro* varies with the temperature at which the measurement is conducted. Not surprisingly, the best correlations with period length *in vivo* are seen at physiological temperatures. In addition, measurements conducted at

different temperatures are correlated, preserving inter-subject differences (Suppl. Fig. 2A). Interesting exceptions exist, however. For example, one subject showed almost perfect temperature compensation at multiple temperatures, in stark contrast to the temperature overcompensation seen in all other subjects (Suppl. Figure 2B). This subject also showed a significant difference between period *in vivo* and period *in vitro* (asterisked subject in Figure 2C). Although the meaning of this difference is uncertain, we suggest that inter-individual genetic variations also exist in the (thus far unknown) loci implicated in temperature compensation by the human circadian oscillator.

In the extreme case, our experiments suggest that a measurement of “true” circadian period emanating from the suprachiasmatic nucleus may be unrealistic. Both *in vivo* and *in vitro*, the observed period appears to be altered by environmental factors. Fibroblast period can be altered by temperature, and physiological period might be sensitive to prior light history and/or retinal function. Nevertheless, measurement of fibroblast period at precise physiological temperatures offers a reasonable approximation of physiological period determined by more rigorous measures. Both values, in spite of their variance, show strong inter-individual differences independent of environment or method, and will likely provide exciting clues to understand the genetic basis of human daily behavior and its influence upon health.

METHODS

Ethical Permission. The study protocol, screening questionnaires, and consent forms were approved by the relevant ethical committee (the Ethical Committee of Basel, Switzerland; the Ethical committee of the Institute of Internal Medicine, SB RAMS, Novosibirsk, Russia; the University of Surrey Advisory Committee on Ethics; and the Moorfields Eye Hospital Ethics Committee) and conform to the Declaration of Helsinki. Informed consent was obtained from all subjects.

Subject selection. Subjects for this study were individuals who have previously or concurrently participated in chronobiological studies of the authors in Basel, Novosibirsk, or Guildford, and were additionally recruited to donate skin biopsies for this study. In two studies (Basel, Novosibirsk), extreme chronotypes were specifically excluded. Where noted, physiological measurements have been reported previously in other contexts. In brief, study participants consisted of nine sighted subjects (6 men, 3 women, mean age ± S.D. 48.3 ± 21.9 years) recruited in Basel, eleven sighted subjects (2 men, 9 women, mean age ± S.D. 25.5 ± 7.8 years) recruited in Novosibirsk, and eight subjects with no perception of light (7 men, 1 woman, mean age ± S.D. 54.6 ± 7.4 years) recruited in Guildford. Further information is cited in Supplementary Methods and in a subject information table included with it, Supplementary Table 1.

Circadian period determination *in vivo*. In order to ensure that the correlations presented herein were not dependent upon a particular method of determining circadian period, each subject group described above participated in a different protocol for the measurement of physiological period *in vivo.* Detailed descriptions of each protocol are listed in Supplementary Methods. In brief, sighted subjects (Basel and Novosibirsk subject groups) were required to adhere to a regular sleep-wake schedule for 5-14 days prior to admission to the laboratory. Subsequently, subjects were maintained under various sets of “constant” conditions designed to eliminate environmental influences upon the circadian oscillator: a 60-hour multiple nap protocol or a 40-hour sleep deprivation protocol (Basel) under constant dimlight (<8 lux) conditions with constrained posture and frequent scheduled meals; or a 4 to 9-day protocol under near-total darkness (<0.1 or 0.2 lux) with normal scheduled sleep episodes (Novosibirsk). Saliva samples were collected at intervals of 0.3-3 hours and analysed for melatonin content through a direct double-antibody radioimmunoassay (Bühlmann Laboratories, Schönenbuch, Switzerland). Blind subjects

(all with no perception of light, Guildford), were studied in their own homes for 3-5 consecutive weeks. No attempt was made to alter the lifestyles of the individuals during the study. For such people, free-running physiological period has been shown previously to remain independent of the solar day even in a home environment [21]. For 48 hours each week, subjects collected sequential ~4-hourly urine samples during the day plus an ~8-hour overnight sample. Urinary 6-sulphatoxymelatonin (aMT6s) concentrations were measured as described previously [29]. Period lengths were determined by the timing of intervals between melatonin rise, either by regression analysis in longer protocols (Novosibirsk, Guildford), or by cosinor analysis for shorter ones (Basel).

Measurement of fibroblast circadian period length. Subjects from each of the studies presented above were recontacted to participate in the present investigation by donating skin biopsies. The time elapsed between measurements *in vivo* and fibroblast donation is indicated in Supplementary Table 1. Detailed methods are presented in Supplementary Methods. Briefly, two cylindrical 2-mm diameter cutaneous biopsies were taken from the buttocks or upper arms of each subject. Fibroblasts were isolated from biopsies and infected using *Bmal1*::luciferase lentivirus as described previously [22]. Five days or more after human fibroblast infection, circadian rhythms were synchronised by 100nM dexamethasone (Sigma), and light output was measured (3 measurements per biopsy; total of 6 measurements per subject) in the presence of 0.1nM luciferin in specially built light-tight atmosphere-controlled boxes for at least 5 days. Period determination was accomplished by cosinor analysis of data between the second and the fifth day of measurement. Values are presented as mean plus or minus standard error from four to eight measurements.

Author Contributions

Experiments for this manuscript were performed in Basel, Zurich, Novosibirsk, and Guildford. Measurements *in vitro* were performed by LP, EAS, EM, FM, and SVC. Human subject recruitment, *in vivo* measurement, and skin biopsy was conducted by VLR, LMH, SWL, JA, JI, and OJS. Data analysis was performed by LP, EAS, EM, VLR, LMH, SWL, CC, and KVD. Drafting and revision of the article were performed on three continents by LP, VLR, SWL, JA, DJS, AWJ, CC, KVD, AE, and SAB, and it took a long time. All authors have finally approved the final version of the manuscript.

Acknowledgements

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Supplementary information

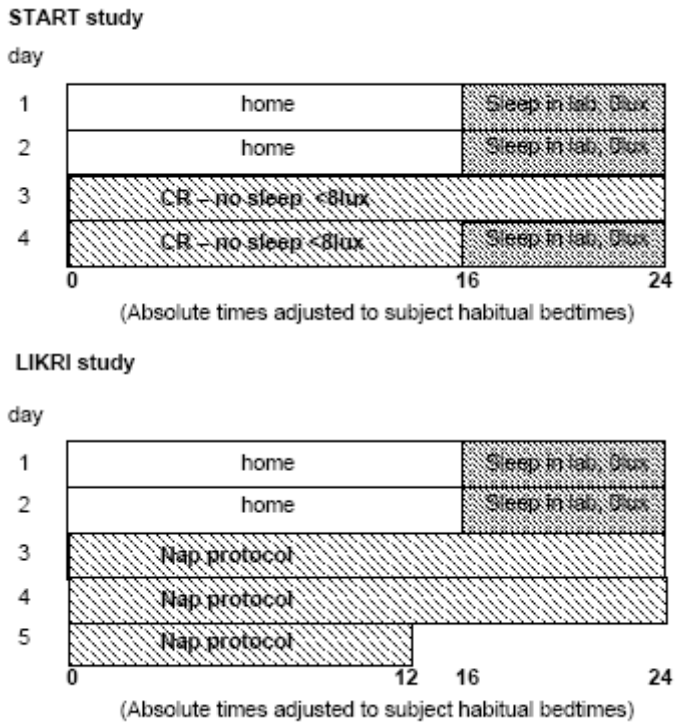
Supplementary methods

Subject selection and ethical permission. The study protocol, screening questionnaires, and consent forms were approved by the relevant ethical committee (the Ethical Committee of Basel, Switzerland; the Ethical committee of the Institute of Internal Medicine, SB RAMS, Novosibirsk, Russia; the University of Surrey Advisory Committee on Ethics; and the Moorfields Eye Hospital Ethics Committee) and conform to the Declaration of Helsinki. Informed consent was obtained from all subjects. Subjects are described in detail in Supplementary Table 1. At the time of *in-vivo* period measurement, all subjects had not taken melatonin or prescribed medications. At the time of skin biopsy, some blind subjects were currently taking melatonin (see Supplementary Table 1). With the exception of one subject (Basel) for whom stable *in-vitro* measurements could not be obtained (Subject Start05), all biopsied subjects were included in our analysis.

Circadian period determination in sighted subjects (Basel). One week prior to the study (baseline week), participants were requested to abstain from excessive caffeine and alcohol consumption (one caffeine-containing beverage per day at most and less than five alcoholic beverages per week). They were instructed to keep a regular sleep-wake schedule during the baseline week at home (bedtimes and wake times within ± 30 minutes of a self-selected target time between 22:00 h and 2:00 h) prior to admission to the laboratory. Compliance was checked by sleep logs and ambulatory activity measurements (wrist activity monitor, Cambridge Neurotechnology Ltd®, UK). Timing of the sleep-wake schedule during the protocol was adjusted to individual average habitual bedtimes. For each participant, habitual bedtime was calculated by centering the approximately 8-hour sleep episodes during the baseline week at their midpoint. The inpatient part of the protocol comprised two baseline 8-hour sleep episodes in darkness in the laboratory, followed by a 40-h sleep deprivation (Start study) or a 60-h multiple nap protocol with the interplay of 16 alternating sleep:wake cycles of 75:150 minutes duration (Likri study). Participants remained under constant conditions (constant dim light levels <8 lux during scheduled wakefulness, semi-recumbent posture in bed, food and liquid intake at regular intervals, no access to time cues) [1,2]. During scheduled sleep episodes a 45 degree shift to a supine posture was allowed, and the lights were turned off (0 lux). Saliva samples were collected every 20 minutes and analysed for melatonin content through a direct

double-antibody radioimmunoassay (validated by gas chromatography–mass spectroscopy with an analytical least detectable dose of 0.65 pm/ml; Bühlmann Laboratories, Schönenbuch, Switzerland). The *in vivo* period length of the sighted subjects was determined via the temporal profile of melatonin content in saliva, using Cosinor^{TM} software (SEPTMR) to fit a hypothetical sinusoidal wave to the collected melatonin data. For the Start study, the temporal profile included the nights before, during, and after the constant routine; for the Likri study, the temporal profiles of the nights before and during the nap protocol were used. Data for one representative subject is shown in Figure 1A.

Suppl. Figure. Protocol for measurement of physiological period in sighted subjects (Basel).

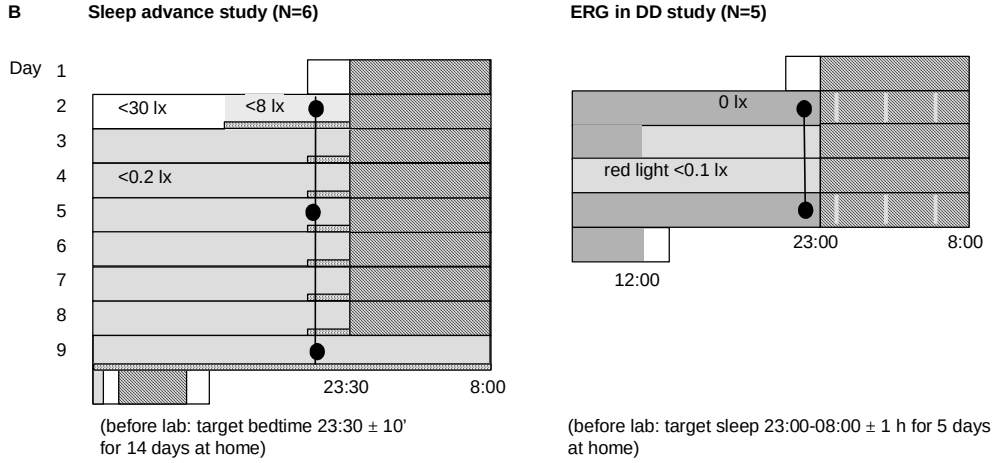


Protocol for two different groups of subjects maintained in either a constant routine (START) or nap protocol (LIKRI). Crosshatched areas are scheduled sleep episodes; stippled areas reflect recumbent awake or nap periods.

Circadian period determination in sighted subjects (Novosibirsk). For the current paper, subjects were recruited that participated in two chronobiological studies during which they adhered to 24-hour days with fixed sleep times in near darkness. The protocols of these studies have been described elsewhere [3, 4]. Generally, the participants were required to go to bed at 23:00-23:30 for 5-14 days prior to admission to the isolation facility. Compliance was checked by wrist actimetry (GähwilerTM, Zurich) and/or sleep logs. The laboratory parts included 24-h days: 9 days under <0.2 lux light ("Sleep-advance" study, #1), or 4 days under <0.1 lux red light ("ERG in DD" study, #2). The saliva

for melatonin assay was collected every 0.5–3 hours on days 2, 5, and 9 during a constant routine protocol in study #1, and on days 1 and 4 in study #2. In the latter study, subjects were additionally awakened to collect night saliva samples three times during both initial and final days of measurements. (These protocols are illustrated in the Supplementary Figure below.) The melatonin content was assayed using the same kits and methods as described for Basel above. All samples from an individual were measured in a single assay. Circadian period in study #1 was obtained by linear regression across times of melatonin evening rise at each of the three days measured. Evening rise was defined as time when melatonin concentration attained 3-pg/ml threshold (by linear interpolation between two 0.5-h-apart values). In the second study, the comparison was made between two times of evening rise (on days 1 and 4) that was computed as the time of the ¼ amplitude crossing after fitting the 24 h melatonin curves with a skewed bimodal baseline cosine function [5].

Suppl. Figure. Protocol for measurement of physiological period in sighted subjects (Novosibirsk). Protocol for two different groups of subjects from Novosibirsk maintained in near-complete darkness (<0.2 lux). Crosshatched areas are scheduled sleep episodes; stippled areas reflect recumbent awake periods.



Circadian period determination in blind subjects (Guildford). The protocols used here to determine physiological period length of blind subjects have been reported elsewhere [6,7,8,9]. Briefly, subjects with no conscious perception of light (NPL) were studied in their own homes for 3-5 consecutive weeks. One had both eyes intact, one had one eye and 6 had no eyes (see Supplementary Table 1). All but one of the blind subjects (#S33)

complained of a sleep disorder as assessed by the Pittsburgh Sleep Quality Index (PSQI; ≥ 5) [10]. No attempt was made to alter the lifestyles of the individuals during the study. For 48 hours each week, subjects collected sequential ~4-hourly urine samples during the day plus an ~8-hour overnight sample. After each collection period, the subjects measured and recorded the volume or weight of the sample and the time of the urine collection period. Urinary 6-sulphatoxymelatonin (aMT6s) concentrations were measured by radioimmunoassay using the method of Arendt et al., [11] adapted by Aldhous and Arendt [12]. Antiserum was supplied by Stockgrand Ltd., University of Surrey. Urinary data (ng/h) from each 48-hour assessment were subjected to cosinor analysis (software provided by Dr. D. S. Minors, University of Manchester, UK) to determine the acrophase, or fitted peak time, of the aMT6s rhythm. The acrophase times were subjected to regression analysis to determine the period (τ = 24 h + slope) [6].

Measurement of fibroblast circadian period length. Subjects from each of the studies presented above were recontacted to participate in the present investigation by donating skin biopsies as described below. The time elapsed between measurements *in vivo* and fibroblast donation is indicated in Supplementary Table 1. During measurements of physiological period length, no subject used medications known to alter circadian phase. At the time of skin biopsy, four blind subjects were concurrently taking melatonin (2-6 mg/day in the evening), one blind participant had recently taken melatonin, and 3 blind subjects reported not taking melatonin. To ensure that this medication did not interfere with measurements *in vitro*, cells from all studies were identically cultivated for at least six weeks prior to measurement, using medium that did not contain melatonin.

Two cylindrical 2-mm diameter cutaneous biopsies were taken from the buttocks or upper arms of each subject. The time of day at which biopsies were taken varied from 10:00 to 14:00, but we have shown previously that time of biopsy does not affect measured period length. [13]. Immediately after removal, biopsies were placed in Dulbecco's Modified Eagle's Medium high glucose (DMEM)/1% Penicillin-Streptomycin solution (Sigma)/1%Glutamax (Sigma), hereafter designated as DMEMc, supplemented with 50% Foetal Bovine Serum (FBS) (Sigma), and shipped on ice to Basel, where cell isolations were conducted. Fibroblasts were isolated from biopsies by digestion of tissue for 4 hours in DMEMc supplemented with 20% Foetal Bovine Serum (FBS) (Sigma) and 87.5 ng/ml liberase (Roche). After digestion, cells were cultured in DMEMc/20%FBS. Confluent cells

were infected using *Bmal1::luciferase* lentivirus as described previously [13]. Five days or more after human fibroblast infection circadian rhythms were synchronized by 100nM dexamethasone (Sigma) in DMEMc + 20% FBS [14]. DMEMc without phenol red was supplemented with 0.1 mM luciferin (Molecular Probes, USA) and light output was measured (3 measurements per biopsy; total of 6 measurements per subject) in specially built light-tight atmosphere-controlled boxes for at least 5 days. For each luciferase measurement, the period of oscillation was calculated by analyzing the period between the second and the fifth day of measurement, fitting hypothetical sine curves with period and phase as free variables using the software Lumicycle Analysis (Actimetrics, USA). The period of the sine wave with the best least-squares fit to the data was assumed to be the true period of oscillation. Values are presented as mean plus or minus standard error from four to eight measurements. A portion of the measures from a representative subject is shown in Figure 1B (the same subject whose in-vivo data are plotted in Figure 1A).

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Supplementary tables

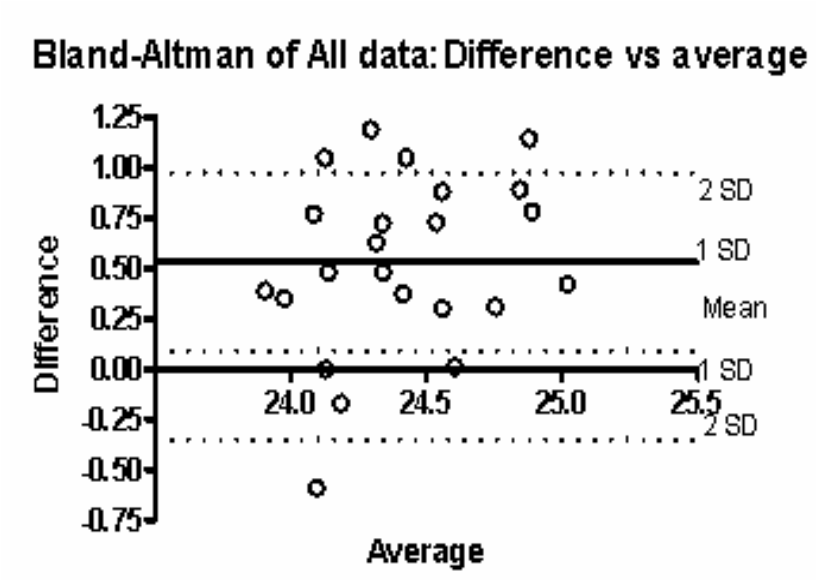
Supplementary Table 1 – Subject Information

Study acronym	Dura-tion	Subject code	Sex	Age at biopsy	Year of study	Year of biopsy	Tau *in vivo*	Tau *in vitro*	Eyes intact
Basel							(cosinor)		
Start (Cajochen *et al.,* 2001; Munch *et al.,* 2005)	3 days	Start 21	m	63	2002	2006	24.45	25.29	2
		Start 23	f	73	2002	2006	24.13	25.01	2
		Start 24	m	64	2002	2006	23.80	23.99	2
		Start 25	m	66	2003	2006	24.10	25.18	2
		Start 31	f	68	2003	2006	24.50	25.26	2
		Start 34	f	63	2003	2006	23.90	25.04	2
Likri		Likri03	m	21	2007	2007	23.90	24.38	2
		Likri04	m	21	2007	2007	23.60	24.65	2
		Likri06	m	21	2007	2008	24.10	24.58	2
Novosibirsk							(regres-sion)		
Sleep-adv (Danilenko et al., 2003)	8 days	cC	m	29	2001	2009	24.08	24.55	2
		Dd	f	32	2001	2009	23.96	24.75	2
		Ee	f	31	2001	2009	24.23	24.60	2
		Gg	m	34	2001	2009	24.00	24.63	2
		Ll	f	42	2001	2009	23.98	24.70	2
		Mm	f	29	2001	2009	23.77	23.90	2
ERG in DD (Danilenko et al., 2009)	4 days	2b	f	50	2004	2009	24.12	25.00	2
		4d	f	22	2005	2009	24.17	24.90	2
		5e	f	22	2005	2009	24.31	25.45	2
		6f	f	22	2005	2009	23.71	24.10	2
		7g	f	23	2005	2009	23.82	23.90	2
Guildford							(regres-sion)		
	3-5 weeks	S23	m	53	1995	2007	24.39	23.80	0
(MT)		S43	m	47	1995	2007	24.13	24.13	0
(MT)		S17	m	57	1994	2007	24.27	24.10	2
		S33	m	67	1995	2007	24.6	24.61	0
		S48	f	66	1995	2007	24.41	24.71	0
(MT)		S45	m	54	1995	2007	24.82	24.17	0
(MT)		S76	m	50	2000	2007	24.6	24.91	1
(MT)		S62	m	49	1996	2007	24.78	25.23	0

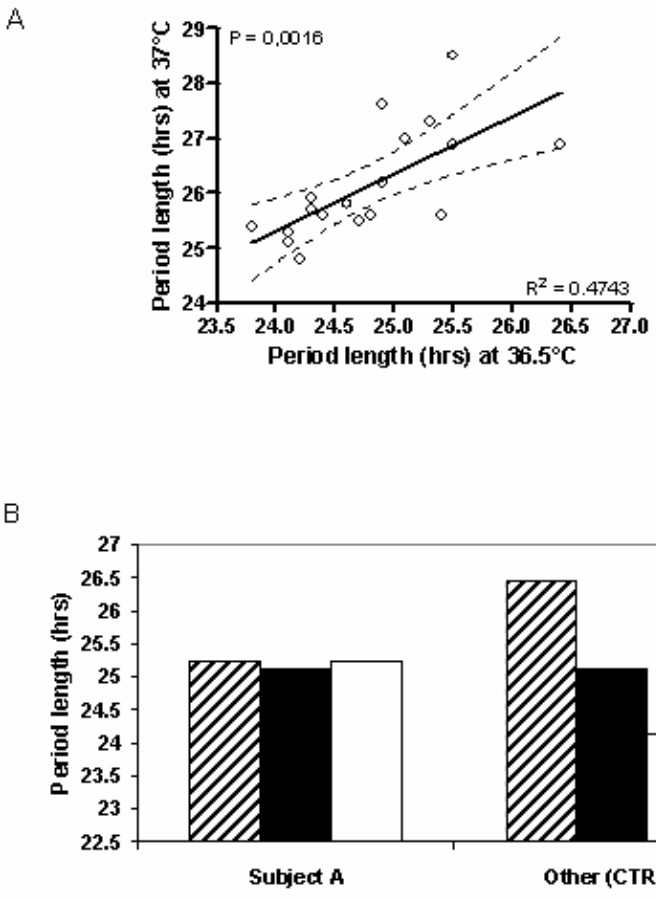
MT – melatonin intake at time of biopsy

Supplementary Table 1 – Subject Information.

Supplementary figures



Suppl. Figure 1. Bland-Altman statistics for period measurements.
For each subject, average period length (*in vivo* and *in vitro*) is plotted against the difference between the two measurements, expressed in standard deviations from the mean.



Suppl. Figure 2. Comparison of fibroblast period length measured at 36.5 degrees C and at 37.0 degrees C incubator temperature.
A. Period length was measured from skin fibroblasts of blind and sighted subjects (from Guildford and Novosibirsk) at two different incubator temperatures, and plotted in comparison. Most subjects showed a similar augmentation in period at the higher temperature (1.1 ± 0.3 hours). B. Extreme temperature compensation properties in one subject – this individual (S45) is asterisked in Figure 2. Fibroblast period lengths at 36.5, 37.0, and 37.5 degrees C are shown for this subject (left) versus another representative subject (S43) (right).

3. A CELLULAR REASON FOR CHANGED DAILY BEHAVIOUR IN THE ELDERLY.

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Submitted (Proceedings of the National Academy of Sciences)

Abstract:

Human ageing is accompanied by dramatic changes in daily sleep-wake behaviour: activity shifts to an earlier phase, and the consolidation of sleep and wake is disturbedAlthough this daily circadian rhythm is brain-controlled, its mechanism is encoded by cell-autonomous circadian clocks functioning in nearly every cell of the body. Therefore, to better understand molecular mechanisms by which human ageing affects circadian clocks, we characterised the clock properties of skin fibroblasts from young and older subjects. Fibroblast period length, amplitude and phase were identical in the two groups, but incubation of these cells with human serum from older donors shortened period length and advanced the phase of cellular circadian rhythms compared to treatment with serum from young subjects. Further experiments demonstrated that this effect is likely to be due to a thermolabile factor present in serum of older individuals. Our results suggest that the molecular machinery of peripheral circadian clocks does not change with age, but some age-related circadian changes observed *in vivo* might be caused by circulating factors.

Introduction

Circadian clocks possess an endogenous periodicity of about 24 hours and play a key role in physiological adaptation to the solar day for all living organisms -- from cyanobacteria and fungi (1) to insects (2) and mammals (3). They influence nearly all aspects of physiology and behaviour, including sleep-wake cycles, body temperature, and the function of many organs (3). During normal ageing, clock function is attenuated, with consequences both for health and quality of life. Older individuals have an earlier phase of everyday activity compared to the young (4). Not only is the consolidation of sleep and wake dramatically reduced (5-6), but overall circadian amplitude of hormones and body temperature is lower (7-8), and many ageing-associated sleep-wake pathologies have been reported (9-11). As a result, one in five healthy older individuals reports taking sleep medications regularly (9). In cases of pathological ageing, chronobiological disturbance is even more acute: Huntington's Disease, Parkinson's Disease, and Alzheimer's Disease are all associated with profound alterations in sleeping patterns (10-12). These effects of ageing on circadian rhythms - diminished circadian amplitude, shorter circadian period, and desynchronisation of rhythms in peripheral organs - have been observed widely in several species of mammals (7, 13-14).

Mammalian circadian clocks are organized in a hierarchical fashion: the suprachiasmatic nuclei (SCN) of the anterior hypothalamus serve as a master clock, receiving light signals from the external environment via the retina and retino-hypothalamic tract, and elaborating these stimuli into signals that are sent all over the body to synchronise clocks in peripheral organs (3). Interestingly, the clock mechanism itself is cell-autonomous, and involves interlocked feedback loops of transcription and translation. These loops are encoded by dedicated clock genes: for example, in one loop the heterodimer formed by the two transcription factors CLOCK and BMAL1 binds cis-acting E-box sequences present in *per* and *cry* gene promoters to activate their transcription. Subsequently, PER and CRY protein complexes inhibit the activity of CLOCK-BMAL1. As a consequence, *cry* and *per* mRNAs decrease in concentration, and a new cycle can start (15).

At a cellular level, both the SCN and peripheral oscillators share the same molecular mechanism (16). Thus, cellular reporters composed of clock gene promoters driving expression of luciferase or green fluorescent protein have proven to be very useful tools for the study of circadian rhythms in the SCN as well as in peripheral oscillators (17-18).

Using such reporters, we have shown previously that many differences in human circadian behaviour can also be seen at a molecular level in peripheral cells. For example, the cellular clocks of early chronotypes (i.e. “larks”) have shorter circadian periods than those of later chronotypes (“owls”) (19), and circadian period length *in vitro* is proportional to physiological period *in vivo* (Pagani *et al*., *PLoS ONE*, accepted). Under entrained conditions in which cellular clocks are constrained to 24 hours, fibroblasts even show the early or late circadian phases of their owners (19).

In this paper we have addressed the effects of ageing on molecular circadian clock properties using a fibroblast-based assay. In principle, alterations in circadian behaviour due to ageing could arise by a variety of mechanisms. Changing neural networks might perturb sleep-wake timing or alter the communication between the SCN clock and other brain regions. Hormonal signals critical for maintaining physiological homeostasis might be perturbed. On a cellular level, molecular changes associated with ageing (e.g. oxidative damage, telomere attrition) might alter basic clock function. Our results are consistent with the hypothesis that the molecular machinery of circadian rhythms in peripheral oscillators is not altered by age, but that molecule/s present in serum might be responsible for some of the circadian changes that occur in the elderly.

Results

Ageing changes human circadian behaviour *in vivo*, but does not alter fibroblast circadian clocks *in vitro*.

To try to understand the molecular changes that might underlie modifications in daily behaviour in elderly individuals, we characterized the circadian rhythms of dermal skin fibroblasts obtained from young and older donors. Subjects were recruited based upon age, but were also asked to give information about daytime preference (their preferred waking time and bedtime both on workdays and during leisure) by completing the Munich Chronotype Questionnaire (MCTQ) (20). The 18 young and 18 older sex-matched subjects participating in our study are described in Table S1.

From the completed MCTQ, older subjects in our study displayed a significantly earlier sleep phase compared to young subjects (Fig.1A; Unpaired t-test $P < 0.01$). This difference reflected well the epidemiological trend that is observed in the general population, e.g. as reported by Roenneberg and colleagues (21). To characterise possible cellular origins of these differences, two 2-mm dermal punch biopsies were taken from every subject. Primary fibroblast cultures were isolated from the biopsies and infected with a lentivirus that harboured a circadian reporter construct (the *Bmal1* promoter driving expression of the firefly luciferase gene (22)). Circadian clocks in infected fibroblast cultures were synchronised with dexamethasone (23), and circadian bioluminescence corresponding to *Bmal1* promoter activity was measured for at least 5 days under constant conditions in a cell culture incubator. The circadian oscillations from fibroblasts from young and elderly subjects were then systematically examined for differences in period length, amplitude and phase. It has been shown previously that chronotype negatively correlates with period length *in vivo* (24) and *in vitro* (19). Hence, if the origins of ageing-related differences were cell-intrinsic, we hoped to see correlations between clock properties *in vitro* and subject age.

The period length for each individual is shown in Fig. 1B. As we have reported for other subject populations (22), fibroblast period differed significantly among different individuals but not between different biopsies from the same individual (Fig. S1). No differences were observed between the groups (Fig. 1B Inset: Unpaired t-test $P > 0.05$; Table S1). Additionally, no correlation was seen between period length and MCTQ sleep phase either

in older or younger subjects (Fig. 1C; Linear regression P > 0.05). (*N.B.* Previous studies showing correlations between questionnaire-based sleep-wake behaviour and period length were based upon comparisons of early vs. late chronotypes. (19, 24)).

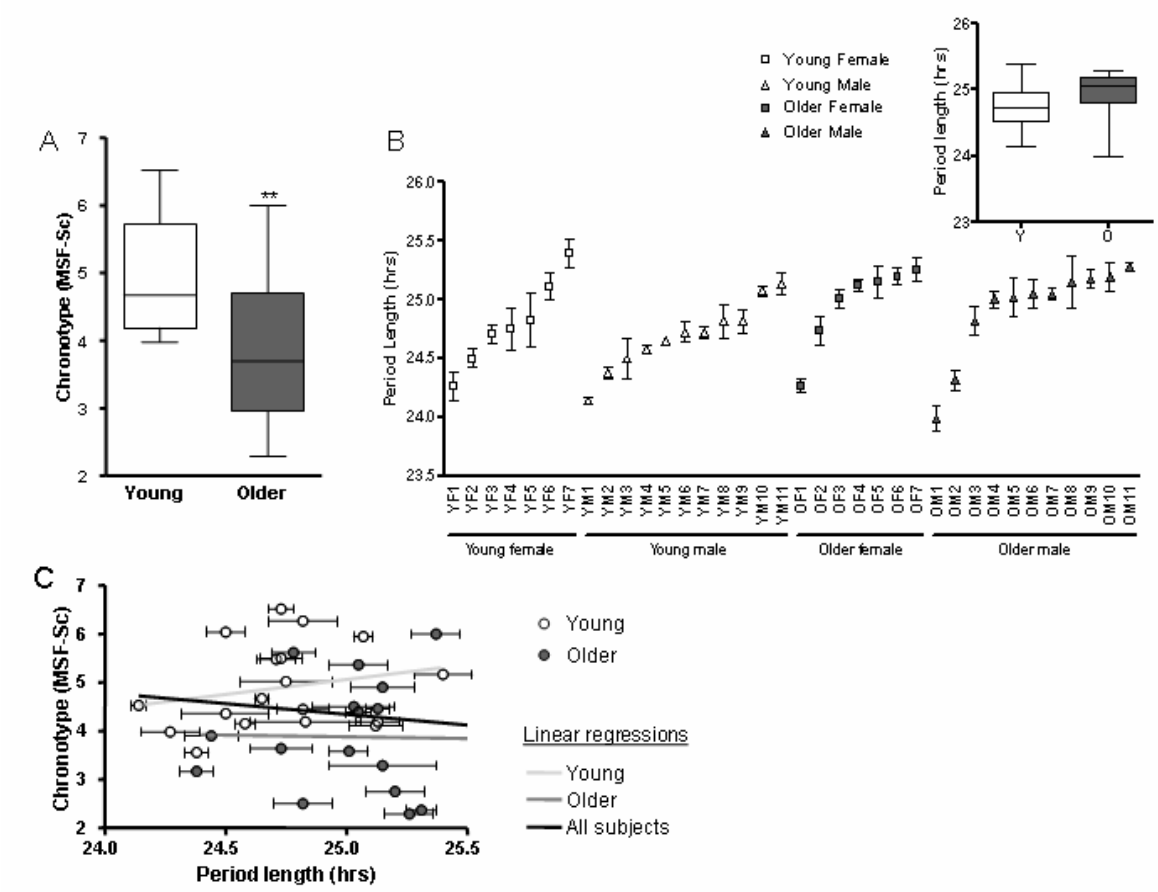

Fig. 1. Influence of age on period length and chronotype. **(A)** Chronotype of young and old subjects, as measured by the Munich Chronotype Questionnaire. The X axis indicates the time of subjects' mid-sleep phase on non-workdays. Dataset variation is shown as a standard boxplot.. N = 18; unpaired t-test **P < 0.01. **(B)** Period length of the primary fibroblasts of each subject participating in this study. For ease of display, data are sorted on the basis of the period length. Data are mean of 6 independent measurements of the period length for every subject ± SEM. **(Inset)** Population average of period lengths of skin fibroblasts from young (Y) and older (O) subjects, shown as a standard boxplot. No statistical difference was observed. N = 18; unpaired t-test P > 0.05. **(C)** Comparison between chronotype and *in vitro* circadian period length for the two groups of subjects (Pall subjects = 0.3707; PY = 0.3785; PO = 0.9229). Period length values are shown as mean of 6 independent measurements ± SEM.

To measure circadian phase in fibroblasts *in vitro*, we entrained fibroblast clocks to a 24-hour daily cycle using periodic oscillations of incubator temperature between 34 and 37 degrees C. After six days, fibroblast daily rhythms entrained well to these cycles in both young and old subjects regardless of their period lengths. On the seventh day, we then measured the phase of reporter gene expression relative to the temperature cycle. An earlier phase was not observed in older vs. younger subjects (Fig. S2A; Unpaired t-test P > 0.05).

Finally, we also studied the circadian amplitude of the oscillations that we observed *in vitro* by measuring the difference between peak and nadir expression values of the second and third cycles, normalized to overall magnitude (Fig. S2B). No correlation to ageing was observed, nor did amplitude correlate with fibroblast cell passage number – i.e. a longer or shorter time in cultivation (Fig. S2C, D; Unpaired t-test $P > 0.05$). Thus, none of the physiological signs of human circadian ageing could be detected or duplicated in cultured fibroblasts from elder subjects.

Human sera influence fibroblast circadian period length and phase.

If cellular circadian properties per se do not change with ageing, but nevertheless these changes are still observed in peripheral organs, hormone levels, or explanted tissues (7, 13-14, 25-26), we reasoned that the age-related alterations might be caused by a circulating factor. To test this possibility, we replaced the normal standardized foetal bovine serum used in our cell cultures with human serum harvested from donors of different ages. The circadian rhythms of 4 young (Y) and 2 old (O) cell lines were measured in the presence of 8 different human serum-containing media from young male donors (YS) and 5 different human serum-containing media from 2 post-menopause female and 3 male older donors (OS). Data regarding these blood donors are listed in Table S2. Fig. 2A and 2B show data of the period length of circadian rhythms from one Y and one O fibroblast cell line, respectively, when measured with each serum, and Fig. 2C and 2D show averages from four different Y lines and two different O lines. Irrespective of whether the treated fibroblasts were from young or older subjects, cells measured in serum from older donors had a significantly shorter circadian period than those in young donors' serum (Fig. 2C-D; Unpaired t-test: $P_Y < 0.001$; $P_O < 0.001$). In the presence of YS, cells from young and older subjects showed a period length of 24.61 ± 0.18 (mean ± SD) and 24.35 ± 0.18 hours respectively, while treating cells from young and older subjects in the presence of OS showed a period length of 23.79 ± 0.16 and 23.60 ± 0.33 hours respectively.

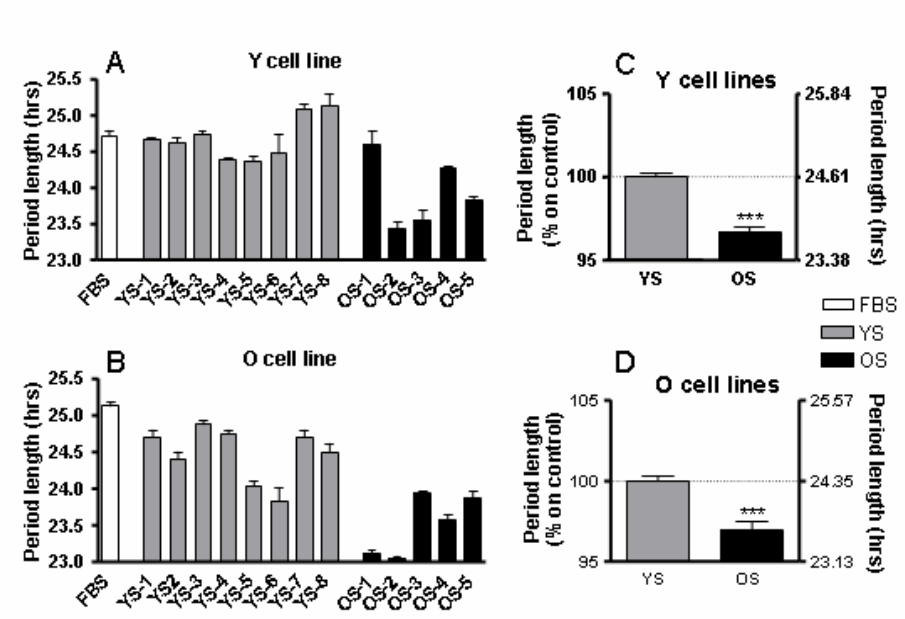

Fig. 2. Circadian period length of skin fibroblasts treated with human serum-containing media. **(A)** Circadian period lengths obtained from one representative cell line taken from a young subject (Y) measured in FBS-containing medium (white), and media containing human serum from 8 young (YS; grey) and 5 older (OS; black) donors. Bars represent the mean of 3 independent measurements ± SEM. **(B)** Equivalent measurements from a representative cell line taken from an older subject (O). **(C)** Graph showing the average period length differences from 4 Y cell lines treated with YS and OS. Left axis, percentage difference; right axis, absolute difference. Results are expressed as average ± SEM. **(D)** Equivalent average for 2 O cell lines. In both cases, treatment of YS gave a highly different period length (Unpaired t-test: ***P < 0.001) compared to the treatment with OS in Y cell lines.

Both *in vivo* and *in vitro*, significant differences in period length have been correlated previously with differences in phase: shorter period lengths lead to earlier phases, both in behaviour and in circadian gene expression (19, 24). To see if the period length differences that we observe between YS and OS could provoke differences in phase, we measured the circadian phase after temperature entrainment of two Y cell lines and two O cell lines in the presence of two YS and two OS. Serum from older subjects indeed phase-advanced the circadian rhythms of cells from both young (Fig. 3A) and older (Fig. 3B) subjects, as compared to the same cells in young serum (Unpaired t-test: $P < 0.05$). Specifically, fibroblasts belonging to young or older volunteers measured in the presence of OS showed a phase advance of 1.96 ± 1.18 hours (mean ± SD) or 2.48 ± 0.46 hours, respectively, compared to the same cell lines measured in the presence of YS.

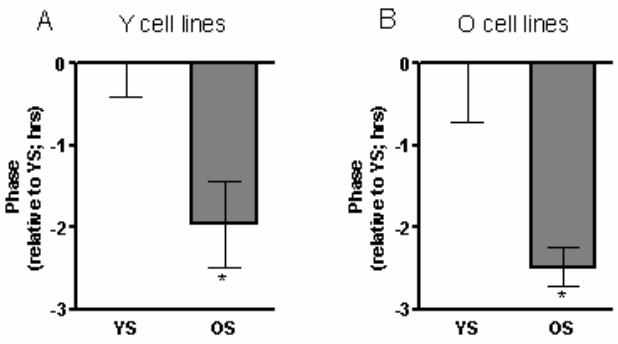

Fig. 3. Circadian phase of fibroblasts in the presence of serum from young and older subjects. **(A)** Phase of cell lines from young subjects (Y) was determined after temperature entrainment in the presence of serum from younger subjects (YS) or from older subjects (OS). Results are expressed as phase difference (in hours) between the two treatments. Each bar results from the average of two different cell lines, each treated with two different sera, ± SEM. **(B)** Equivalent data using cell lines from older subjects. In both cases, there was a significant advance in the phase of cells treated with OS compared to the same cell lines treated with YS (Unpaired t-test: *P < 0.05).

The influence of sera from older subjects on period length is due to heat-sensitive substance/s.

Our results above suggest that one or more substances in human serum can recapitulate at a cellular level the differences in circadian phase seen between younger and older subjects. Such effects could in principle arise from substances either in YS or in OS. To investigate further the nature of the substance/s responsible for the ageing effects, we heat-inactivated 4 YS (YSHI) and 4 OS (OSHI). In this way the secondary structure of proteins and unstable metabolites would be destroyed. The circadian period length from 2 Y and 2 O cell lines were analysed in the presence of medium containing each of these sera (Fig. 4 A-B and Suppl. Fig. 3A-B; average values in Fig. 4C-D). Although measurements from cells with YSHI were not different compared to measurements with YS (period Y = 24.61 ± 0.18 hours (with YS, mean ± SD) versus 24.63 ± 0.27 hours (with YSHI); period O = 24.35 ± 0.18 (with YS) versus 24.50 ± 0.45 (with YSHI); PY > 0.05; PO > 0.05), measurements with OSHI were significantly longer than with OS, and not significantly different from those with YS. (period Y = 23.79 ± 0.16 (with OS, mean ± SD) versus 24.60 ± 0.14 (with OSHI); period O = 23.60 ± 0.33 (with OS) versus 24.32 ± 0.28 (with OSHI); one-way ANOVA Tukey's Multiple Comparison test: PY < 0.001; PO < 0.01). Thus, longer period was rescued by the heat-inactivation of OS, suggesting that the substance(s) responsible for the effects that we observe are heat-sensitive circulating factors present in serum from older individuals.

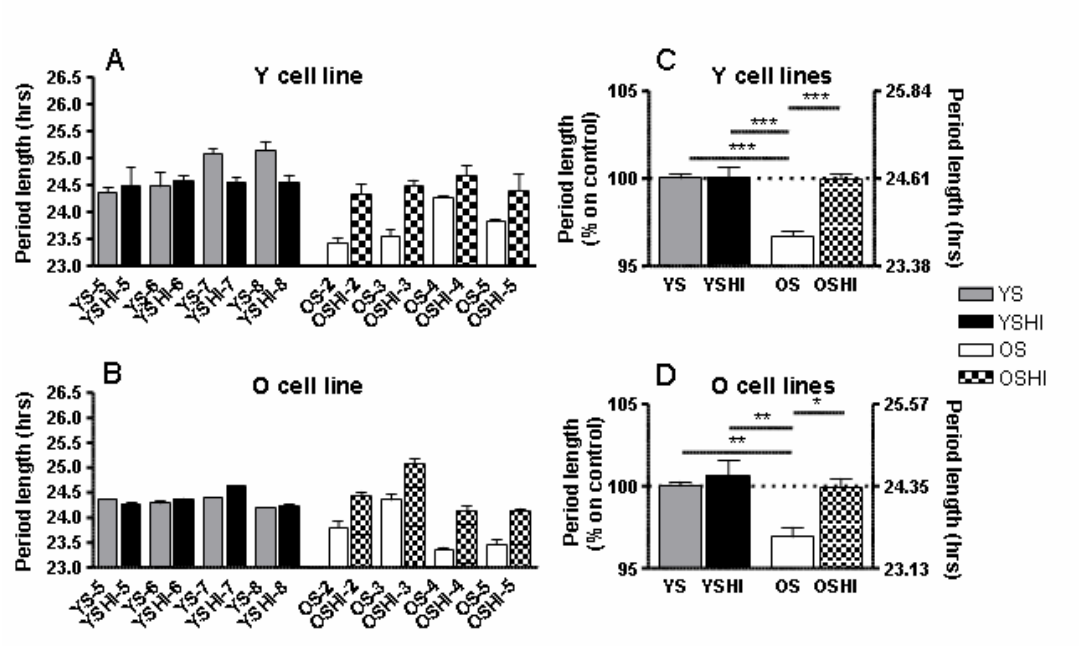

Fig. 4. Circadian period length of skin fibroblasts treated with normal and heat-inactivated human serum-containing media. **(A)** Circadian period length obtained from one representative cell line belonging to a young subject (Y) measured in media containing normal or heat-inactivated human serum from 4 young and 4 older donors. Every bar is the mean of 3 independent measurements ± SEM. **(B)** Equivalent measures from a representative cell line from an older subject (O). **(C)** Graph showing the average period length from Y cell lines treated with normal and heat-inactivated serum from both young (respectively YS and YSHI) and old subjects (respectively OS and OSHI). Each bar represents the average of 2 different cell lines, each treated with 4 different sera, ± SEM. Left axis, percentage difference; right axis, absolute difference in hours. **(D)** Equivalent graph for cell lines from older subjects. In both cases, YSHI did not modify the period length compared to YS (One-way ANOVA Tukey's Multiple Comparision Test: $P > 0.05$), whereas OSHI increased the period length compared to OS (One-way ANOVA Tukey's Multiple Comparision Test: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) to a length equivalent to that obtained with YS (One-way ANOVA Tukey's Multiple Comparision Test: $P > 0.05$).

Theoretically, either a diurnal factor or one constantly present could achieve the effects that we observe. Multiple phase-shifting activities are known to be present in serum, no matter what time of day it is drawn (23). Two obvious labile circadian candidates would be melatonin and cortisol, hormones widely used as circadian markers and known to phase-shift the circadian clock. Therefore, we tested the levels of these two substances explicitly in our sera: they were not significantly different between young and older blood donors at the time that these sera were taken (ca. 2 p.m.) (Fig. S4A-B; Unpaired t-test: $P > 0.05$). Melatonin is barely present in serum at these times. Although a trend toward elevated cortisol is visible in older subjects, fibroblasts treated with different amounts of cortisol at levels found in the blood of these donors did not show differences in period length compared to untreated cells (Fig. S4C).

Discussion

In this study, we have shown that whereas skin fibroblasts taken from young and older subjects do not differ *per se* in their circadian properties, incubation of both cells in serum from older subjects results in a shortening of circadian period and a shift to earlier phase compared to incubation with serum from younger subjects. Moreover, the effects that we observe are due to a thermolabile activity in older serum.

Although circadian and sleep-wake disruptions have been documented extensively in elderly individuals, the causes of these changes are not well understood. According to one theory, fragmentation of the sleep-wake cycle coupled with increased daytime napping results in less nighttime sleep and a shift to an earlier activity phase (26-27). Thus, age-related reduction in the evening circadian signal that opposes homeostatic sleep pressure modifies sleep structure and sleep consolidation (28-29). Moreover, changes in eye physiology with age (lens yellowing and senile miosis, cataracts, etc.) and behaviourally, more time indoors are thought to reduce the entraining effects of solar light, further exacerbating the problem (30). Recent studies corroborate these hypotheses, and demonstrate clearly that the circadian system is less sensitive to light in the elderly (31-32).

The results that we present provide an additional cellular explanation for the shift toward earlier chronotype occurring in elderly individuals: a hormonal factor might both shorten period and advance phase. Indeed, at a behavioural level, both period shortening and phase-shifting have been observed in older animals (33-34). Problematically, however, in human studies of circadian period *in vivo* under “forced desychrony” laboratory conditions that permit the measurement of circadian period independent of sleep-related input, no changes in period have been observed (26, 35). In these same studies, a shift in relative sleep phase is still present. Thus, these human studies have documented a change in phase *without* a corresponding change in period.

We think that this apparent contradiction can be explained in light of the hierarchical structure of the mammalian circadian system. If a circulating clock-entraining substance that shortened period were *unable to entrain* the master pacemaker in the SCN, but affected other oscillators, the result would be a shift in phase of many clock-controlled functions *without* a change in SCN-controlled period. The observed effect would be

mechanistically akin to the way that individuals of shorter and longer intrinsic period entrain to the 24-hour solar day by showing earlier and later phases of behaviour. The putative causal factor could be constantly present in serum, and use asymmetry in phase response curves to shift overall phase. Alternatively, it could be secreted at a particular time of day. This factor might be present uniquely in elderly, or it might be a factor present in both older and younger individuals, but phase-shifted in its expression.

Our results suggesting the existence of such a factor are based purely upon *in vitro* assays. Further studies are clearly needed to identify the substance or substances that are responsible for the period and phase differences that we observed, as well as how ageing alters their production and whether antagonists of their effects might provide real benefits to aged individuals. The findings open the possibility that circadian difficulties associated with ageing might be pharmacologically treatable without recourse to potentially addictive sleep aids, and this would represent a major benefit to health.

Materials and methods

Subject recruitment criteria. Eighteen healthy young (ages 21-30 years old) and eighteen healthy older subjects (ages 60-88 years old) were chosen for participation based upon age alone, and were asked to fill out the Munich Chronotype Questionnaire (MCTQ) to determine their sleep phase (20). Subject statistics are shown in Supplementary Table 1. Prior ethical consent for the use of human skin tissues was given by the Ethical Committee of Basel, and informed written consent for participation in this study was obtained from all human subjects.

Tissue isolation, Fibroblast Culture and Viral Infection. Two cylindrical 2-mm diameter cutaneous biopsies were taken from the buttocks of each recruited healthy subjects. Fibroblasts were isolated from biopsies by 4 hours digestion of tissue in Dulbecco's Modified Eagle's Medium high glucose (DMEM)/1% Penicillin Streptomycin (Sigma, USA)/1% Glutamax (Sigma, USA) (DMEMc)/20% Foetal Bovine Serum (FBS) (Sigma, USA)/87.5 ng/ml liberase (Roche, USA), and cultured in DMEMc/20% FBS. Confluent cells were infected using *Bmal1::luciferase* lentivirus. Three days after infection transfected cells were positively selected (22).

Harvesting of sera. At 2 p.m. 45 ml of blood were collected from 8 healthy young (8 male; age 25.5 ± 4.6 years) and 5 healthy older (3 male and 2 post-menopause female; age 74.4 ± 9.8 years) subjects in clot activator vacutainers (BD Vacutainer System, UK). Whole blood was incubated 30 minutes at room temperature and the centrifuged 10 minutes at 2000 g. Serum was harvested and stored at -20° C. W hen specified, human serum was heat-inactivated by treatment for 30 minutes at 56° C.

Synchronisation and measurement of circadian period and phase. Five days or more after human fibroblast infection circadian rhythms were synchronised by 100 nM dexamethasone (Sigma, USA) in DMEMc + 20% FBS (36). DMEMc without phenol red was supplemented with 0.1 mM luciferin (Molecular Probes, USA) to obtain the counting medium (CM) and light output was measured in homemade light-tight atmosphere-controlled boxes for at least 5 days. For the measurements of fibroblast basal circadian rhythms CM was supplemented with 10% FBS; for the influence of human serum on circadian period length CM was supplemented with 10% human serum; for determination of the influence of cortisol on period length CM was supplemented with 10% sFBS, or 10%

sFBS and 25 ng/ml cortisol (Sigma, USA) or 10% sFBS and 75 ng/ml cortisol; for determination of the influence of heat-inactivated human serum on period length CM was supplemented with 10% heat-inactivated human serum. For phase determination experiments, cells in luciferin-supplemented medium were synchronised by incubation for 6 days in a temperature-controlled incubator under a 16h : 8h 35° C : 37° C daily temperature cycle. On day seven, cells were transferred to the Lumicycle device at 37° C and bioluminescence was measured for an additional 16 h. Cellular phase was determined by measuring the time of the transcriptional maximum of reporter gene expression in the smoothed and normalized dataset during this interval (19).

Melatonin determination in sera. A direct double-antibody RIA was used for the melatonin assay, validated by gas-chromatography-mass spectroscopy (Buehlmann Laboratories, Schoenenbuch, Switzerland). The minimum detectable dose of melatonin (analytical sensitivity) was determined to be 0.2 pg/ml. The functional least-detectable dose using the less than 20% coefficient of interassay variation criterion was less than 0.65 pg/ml. Melatonin concentrations in the sera were expressed as pg/ml of serum.

Cortisol determination in sera. Quantification of cortisol in sera was performed using a Cortisol ELISA Kit (R&D, UK) following the manufacturer's instruction. Cortisol concentration in the sera was expressed as ng/ml of serum and the assays were performed in duplicate.

Statistical methods (period). For each luciferase measurement, the period of oscillation was calculated by fitting the curve to sine waves of known period using a macro program for Microsoft Excel written by SAB. The maxima and minima of each oscillation were identified, and the timing of these points was used to fit hypothetical sine curves with period and phase as free variables. The period of the sine wave with the best least-squares fit to the data was assumed to be the true period of oscillation. Because the period length of the first day after synchronisation varied according to the conditions of synchronisation, it was not included in these calculations; rather, period was determined by analyzing only days 2–5. To determine the period length of a particular biopsy three separate experimental measurements were done for each fibroblast culture. To determine the period length of a particular individual, at least two separate biopsies were analyzed in this manner. Values are presented as mean plus or minus the standard error.

Statistical methods (relative amplitude). The amplitudes of the second and third cycles of circadian expression were obtained as the difference between the peak and nadir expression values; these measurements were then normalized using the magnitude of the first peak, to eliminate the bias due to the fact that the magnitude of the bioluminescent signal measured is proportional to the reporter virus infection efficiency in our fibroblasts.

Acknowledgements

This project was supported by grants from EUCLOCK from E.U. #LSHM-CT-2006-018741, from the Désireé & Niels Yde Foundation, from the Swiss National Science Foundation #310000-122572 and #31003-113874 and from the Synapsis Foundation. Thanks to Robert Dallmann for critical reading of this manuscript.

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Supplementary information

Supplementary tables

Table S1. Subject Characterization

	N	Age (years ± S.D.)	Chronotype (MSF-Sc ± S.D.)	Fibroblast period length (hrs ± S.D.)
Young	18	25.44 ± 3.58	4.98 ± 0.84	24.73 ± 0.32
YF	7	25.86 ± 2.48	4.86 ± 0.78	24.80 ± 0.38
YM	11	25.18 ± 4.24	5.06 ± 0.91	24.69 ± 0.29
Older	18	67.89 ± 7.32	3.88 ± 1.12	24.94 ± 0.37
OF	7	65.43 ± 4.12	4.01 ± 1.22	24.96 ± 0.35
OM	11	69.45 ± 8.61	3.79 ± 1.10	24.92 ± 0.40

Table S2. Blood Donor Characterization

Blood donors	Gender	Age	Medicaments
YS-1	M	21	No
YS-2	M	20	No
YS-3	M	32	citalopram (120 mg/day)
YS-4	M	21	No
YS-5	M	26	No
YS-6	M	27	No
YS-7	M	31	No
YS-8	M	26	No
OS-1	M	81	diosmine (450 mg/day)/Esperidine (50 mg/day)
OS-2	F	83	Aspirine cardio (100 mg/day); Metoprolol (50 mg/day); Perindopril/indapamin (4 mg/day/1.25 mg/day)
OS-3	F	67	No
OS-4	M	80	Aspirine cardio (100 mg/day); Amlodipine maleate (2.5 mg/day); Chlorthalidone (12.5 mg/day)
OS-5	M	61	Aspirine cardio (100 mg/day); Simvastatine (40 mg/day)

Supplementary figures

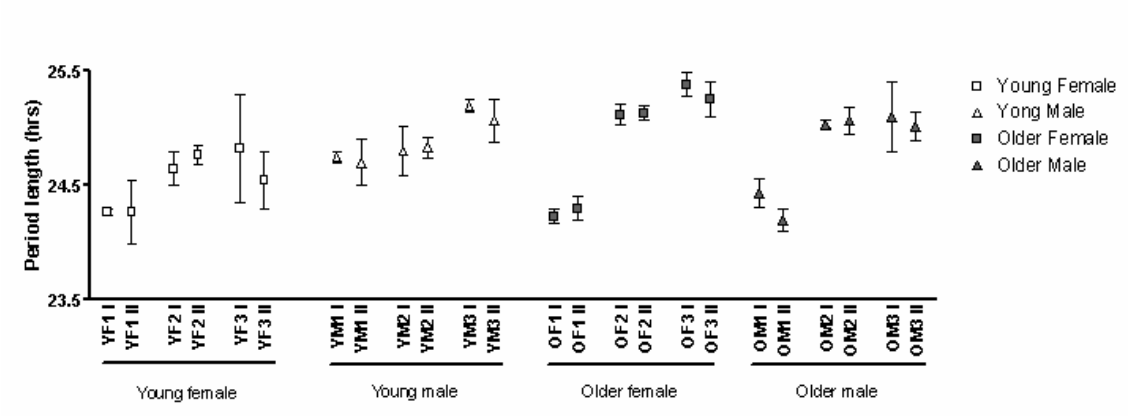

Fig. S1. Period lengths obtained from two different biopsies taken from 3 young females, 3 young males, 3 older females and 3 older males. Each bar represents the mean of 3 independent measurements per biopsy ± SEM.

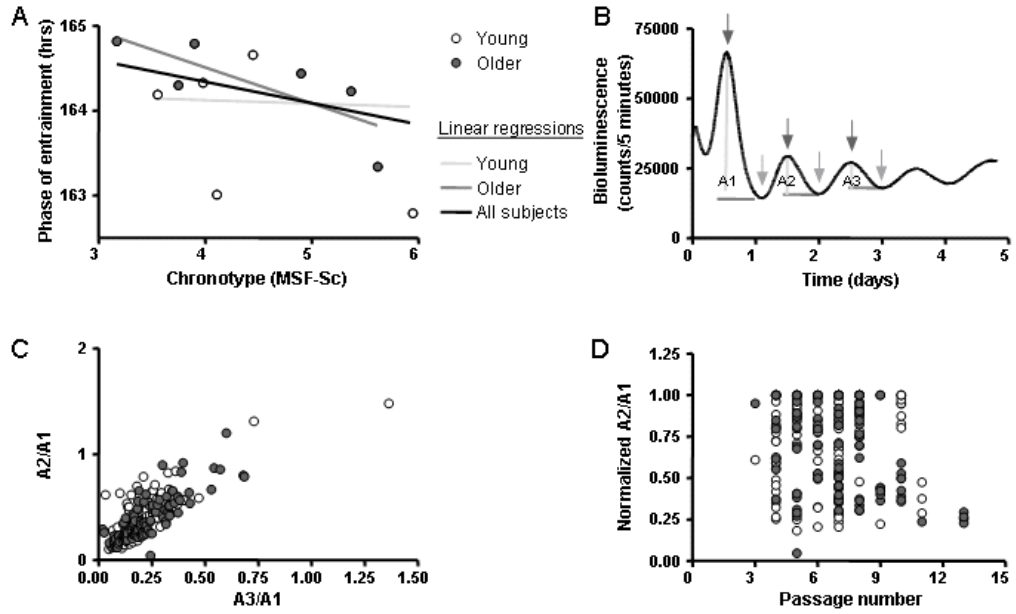

Fig. S2. Influence of age on amplitude and phase of entrainment. **(A)** Graph showing chronotype *vs.* phase of entrainment of reporter expression for fibroblasts from 5 young and 6 older subjects. Statistical analyses revealed no significant correlation between phase of entrainment, chronotype and ageing ($P_{All\ subjects}$ = 0.3699; P_Y = 0.9462; P_O = 0.0698). **(B)** Schematic representation of the amplitude measurement. Amplitude of the first three peaks (first peak = A1; second peak = A2; third peak = A3) was measured. Damping was approximated as the ratio A2/A1 or A3/A1, which produced equivalent results. (See description in Materials and Methods.) **(C)** Diagram showing the correlation between A2/A1 and A3/A1 for young and old subjects. Age did not influence either of these ratios (Unpaired t-test: P > 0.05). **(D)** Graph reporting the influence of cellular senescence on the damping rate of peripheral oscillators. Abscissa, passage number of the measured cells; ordinate, damping rate A2/A1, normalized to the highest value obtained from each subject. There was no difference in amplitude of fibroblasts between young and old subjects with increasing passage number (Unpaired t-test: P > 0.05).

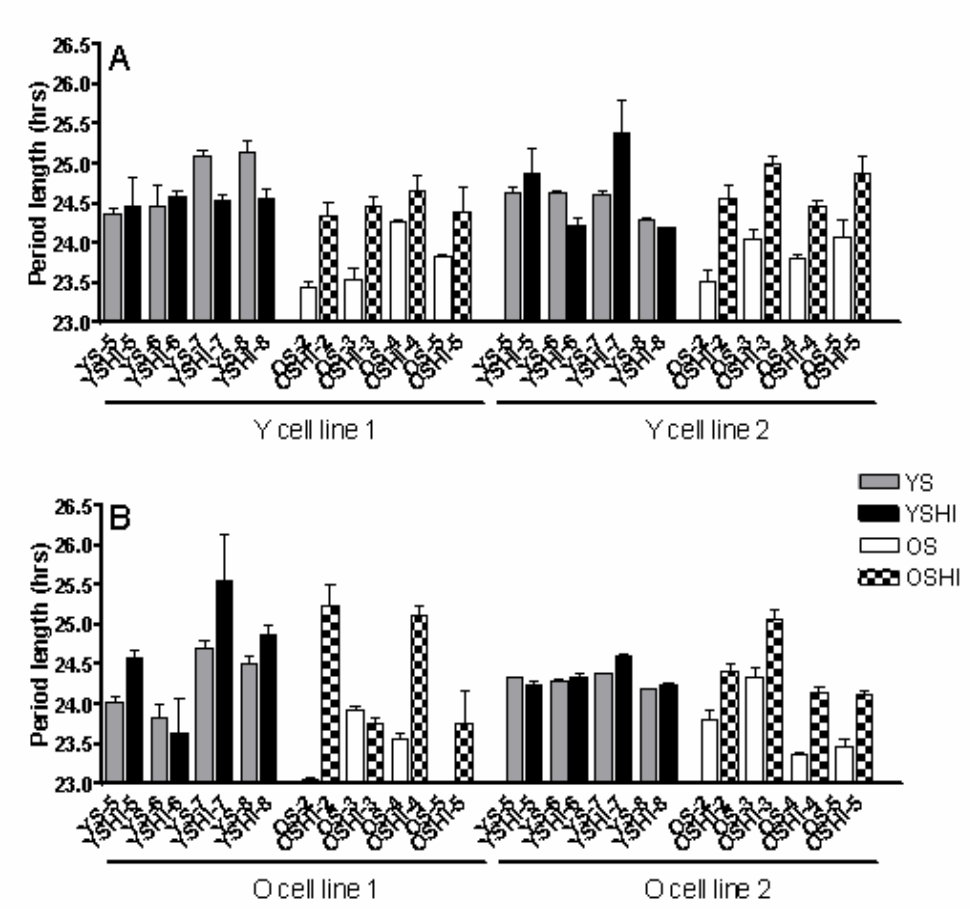

Fig. S3. Details of the circadian period length of skin fibroblasts treated with normal and heat-inactivated human serum-containing media. **(A)** Circadian period length obtained from cell lines belonging to 2 young subjects (Y) measured in media containing normal or heat-inactivated human serum from 4 young and 4 older donors. Every bar is the mean of 3-6 independent measurements ± SEM (exceptions: for Y1-OSHI5 and Y2-OS2, n=2). **(B)** Equivalent measures from 2 lines from older subjects (O). Bars are the mean of 3 independent measurements ± SEM. (exception: for O1-OSHI2, n=2).

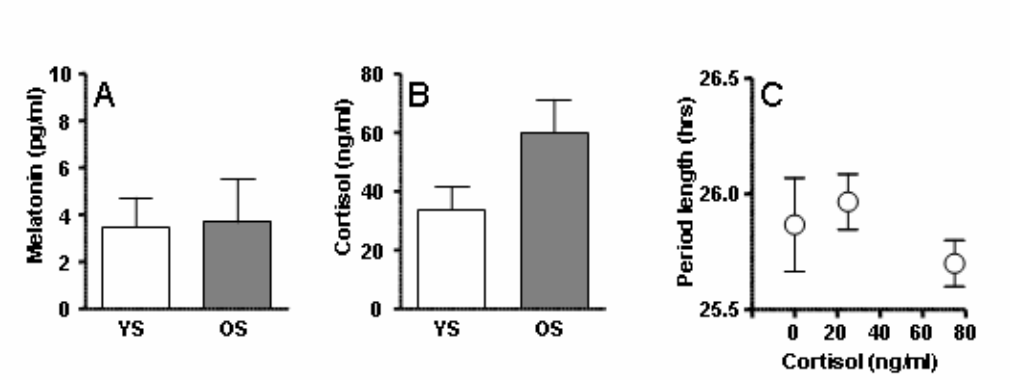

Fig. S4. Levels of melatonin and cortisol in 4 sera from young donors (YS) and 4 sera from older donors (OS). **(A)** Melatonin levels (pg/ml) in 4 YS and 4 OS. There was no statistical difference in melatonin concentration between the two groups of sera (Unpaired t-test: $P > 0.05$). **(B)** Cortisol levels (ng/ml) in 4 YS and 4 OS. There was no statistical difference of cortisol concentration between YS and OS (Unpaired t-test: $P > 0.05$). Since a (non significant) trend was visible, the effects of cortisol upon period length were studied in more detail in the next panel. **(C)** Period lengths of one cell line in serum supplemented with 0 ng/ml, 25 ng/ml, or 75 ng/ml cortisol. Every point represents the mean of 3 independent measurements ± SEM . No significant differences were observed (Unpaired t-test: $P > 0.05$).

4. NO EVIDENCE FOR A DIRECT “ZEITGEBER” ROLE OF MELATONIN IN HUMAN PERIPHERAL CLOCKS.

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Submitted (Nature Neuroscience)

Abstract:

Melatonin affects SCN activity and synchronizes most of the animal and human sleep-wake cycle. Here we used human skin fibroblasts as model to investigate the zeitgeber properties of melatonin on peripheral oscillators. Melatonin could not restore the rhythms of damped fibroblasts, rather it reduced the bioluminescence emitted by newly synchronized cells, excluding a direct effect of melatonin as peripheral oscillator zeitgeber.

Manuscript:

During the dark period of the day, the pineal gland produces melatonin in response to a multisynaptic signal involving several areas in the central nervous system including the circadian master clock suprachiasmatic nucleus. Melatonin pathways are propagated mainly through high affinity G-coupled receptors MTNR1A and MTNR1B[1]; these receptors are expressed in skin[2] as well as in other tissues (for a review see [3]). Several studies indicate that melatonin has synchronizing properties on human activity rhythms[4] and rodents sleep-wake cycles[5] likely by modulating SCN firing rate[6]. Moreover, the circadian presence of melatonin in peripheral biological fluids (blood, saliva and urine) suggests that melatonin could be an important signal that the SCN might use to synchronize non-neuronal peripheral oscillators.

In this experiment we investigated this hypothesis by using skin fibroblasts isolated from punch biopsies as a cellular model of peripheral oscillators.

We first confirmed the expression of melatonin receptors mRNA and protein expression in human fibroblasts (**Fig. 1a,b**).

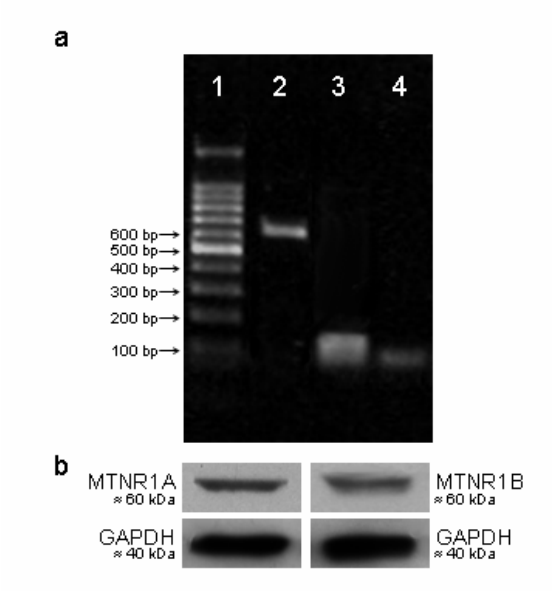

Fig. 1. Human skin fibroblasts express G-coupled melatonin receptors. (**a**) Picture of PCR products on an agarose gel: DNA ladder, 100 pb, 1; MTNR1A 570 bp, 2; MTNR1B 118 bp, 3; CDK4 64 bp, 4. (**b**) Expression of melatonin receptors protein MTNR1A (about 60 kDa) and MTNR1B (about 60 kDa) and the internal control GAPDH (about 40 kDa) in skin fibroblasts.

Subsequently, cells were transfected with the reporter gene *Bmal1::luciferase* for the real-time detection of circadian rhythms[7]. We wished to investigate whether melatonin might resynchronize the circadian rhythms of damped fibroblasts, as dexamethasone or changing medium. After the pre-synchronization of circadian rhythms and 3 complete daily

cycles of *Bmal1* expression, cells were treated with vehicle (V), positive control (PC) (dexamethasone $0.1*10^{-6}$M), or melatonin at increasing concentrations (from $2*10^{-11}$ M to $5*10^{-4}$ M) at different time-points (at CT 6, CT, 12 and CT 24, considering CT 0 the maximum activity of luciferase after 3 days) to test the time-dependent synchronizing effects of the drugs (**Fig. 2a inset**). In case of a re-synchronization of the rhythms either a phase change or an increasing of the amplitude of bioluminescence may be observed. No difference in the amplitude of the cycle before the treatments (A3) were found (data not shown; One-way ANOVA over all data: CT 6 P = 0.9399; CT 12 P = 0.4907; CT 24 P = 0.6262). A good re-synchronization was obtained after the PC application (**Fig. 2a**) (mean ± SD: CT 6 $_{PC}$ = 434.60 ± 104.80; CT 6 $_{V}$ = 100.00 ± 46.04; P $_{CT\ 6}$ < 0.01; CT 12 $_{PC}$ = 461.00 ± 324.50; CT 12 $_{V}$ = 100.0 ± 25.47; P $_{CT\ 12}$ < 0.01; CT 24 $_{PC}$ = 364.80 ± 153.60; CT 24 $_{V}$ = 100.00 ± 66.90; P $_{CT\ 24}$ < 0.001; two-way ANOVA P $_{treatment}$ < 0.0001, P $_{CT}$ = 0.0574, Bonferroni post hoc test to compare replicate means by row, versus V treatment; α = 0.05), whereas the treatments with melatonin did not synchronize human fibroblasts at any time points and concentrations (P > 0.05: two-way ANOVA, Bonferroni post hoc test to compare replicate means by row, versus V treatment) (**Fig. 2a-d**). Similar results of melatonin were found using another cell line (**Fig. 2e, inset**) (One-way ANOVA P = 0.0507, Bonferroni post hoc test to compare all pairs of columns, versus V: P > 0.05; α = 0.05).

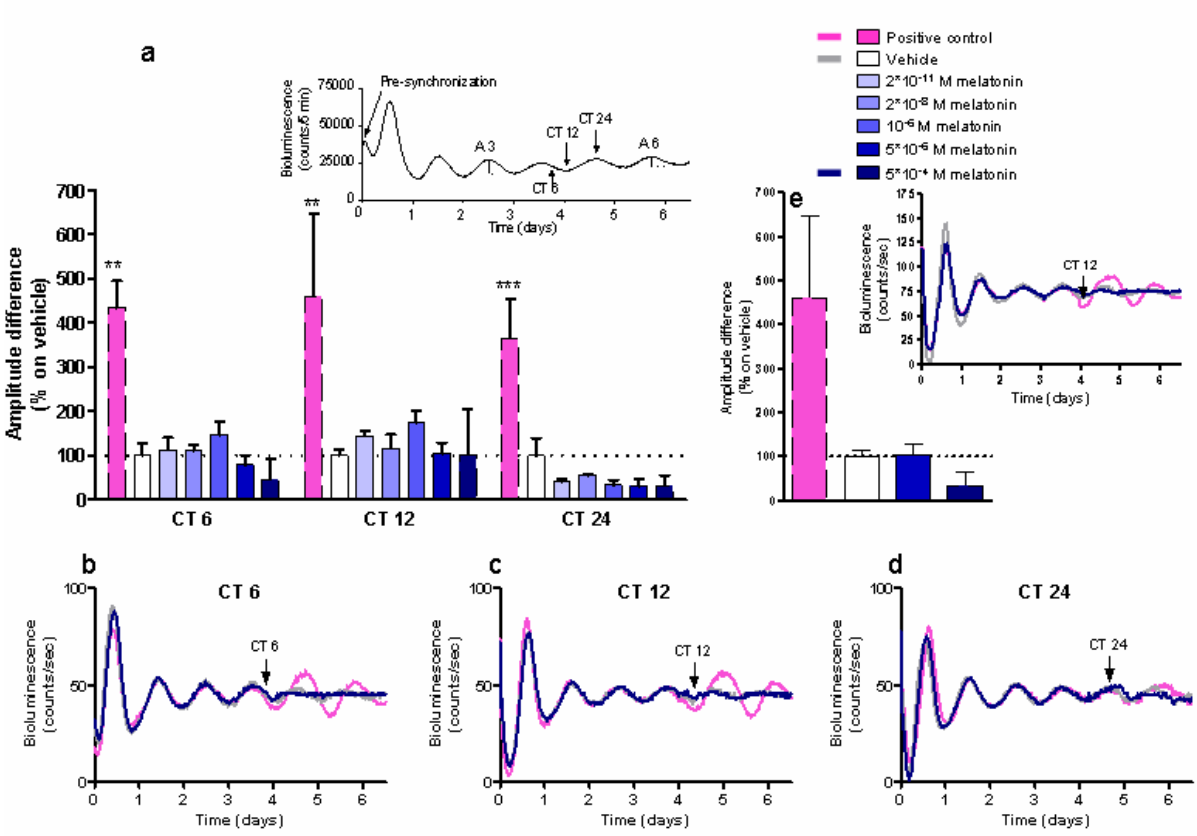

Fig. 2. Effect of a single dose of melatonin administration on the amplitude of the reporter gene luciferase under Bmal1 promoter in human skin fibroblasts. (**Inset**) Experimental design. For details see supplementary methods. (**a**) At the tested concentrations melatonin did not induce synchronization, unlike positive control (PC) treatment, compared to vehicle (V) treatment. Data are plotted as the mean of 3 independent measurements ± SE. ** P < 0.01; *** P < 0.001. (**b-d**) Representative bioluminescence measurement diagrams using V (in grey), PC (in violet) or melatonin ($5*10^{-4}$ M, in blue) treatment at CT 6 (**b**), CT 12 (**c**) and CT 24 (**d**). (**e**) Lack of synchronizing effect of a single melatonin administration on human skin fibroblasts from a second subject. Each bar represents the mean of 3 independent treatments ± S.E.. (**Inset**) Representative bioluminescence measurement diagram using V (in grey), dexamethasone (in violet) or melatonin ($5*10^{-4}$ M, in blue) treatments at CT 12.

The analyses of the phase of entrainment revealed that only PC administered at CT 12 phase delayed the rhythms (mean ± SD: CT 12 $_{PC}$ = -6.130 ± 0.7071; CT 12 $_{V}$ = 1.987*10-8 ± 1.260; P $_{CT\ 12}$ < 0.01; two-way ANOVA P $_{treatment}$ = 0.0011; P $_{CT}$ = 0.2299, Bonferroni post hoc test to compare replicate means by row, versus V; $\alpha = 0.05$), although a tendency of PC and melatonin $5*10^{-4}$ M administered at CT 24 to respectively phase delay and phase advance the rhythms was observed (unpaired t-test: respectively P = 0.0585; P = 0.0623; $\alpha = 0.05$) (**Fig. 3a**).

To confirm the absence of zeitgeber properties of melatonin on peripheral oscillators, human fibroblasts were washed once and then treated with medium containing luciferine alone (L0) or luciferine and $5*10^{-4}$ M melatonin (ML0). Measurements of the bioluminescence at the zenith peak at nadir peak of the first 3 cycles revealed that

fibroblasts treated with ML0 emitted less bioluminescence compared to cells treated with L0 (**Fig. 3b**) (1 zenith peak $P < 0.001$; 2 zenith peak $P < 0.05$; two-way ANOVA $P_{treatment} < 0.0001$; $P_{peaks} < 0.0001$, Bonferroni post hoc test to compare replicate means by row. 1 nadir peak $p = 0.0389$, unpaired t-test; $\alpha = 0.05$). To exclude that the previous result was due a decreased viability of luciferine caused by a chemical reaction with melatonin[8], fibroblasts were washed once and then treated with a medium containing luciferine alone (L6) or luciferine and $5*10^{-4}$ M melatonin (ML6) previously incubated for 6 days at 37℃ and 7.5% CO_2. The analyses of the light emitted by the cells revealed that fibroblasts treated with L0 and L6 emitted the same amount of light, as well as cells treated with ML0 and ML6 (**Fig. 3b,inset**) (mean ± SD: L0 = 145.80 ± 10.12; L6 = 161.00 ± 32.99; ML0 = 103.10 ± 6.71; ML6 = 124.00 ± 30.26; unpaired t-test: $P_{L0-L6} = 0.4874$; $P_{ML0-ML6} = 0.3066$; $P_{L0-ML0} = 0.0037$; $\alpha = 0.05$).

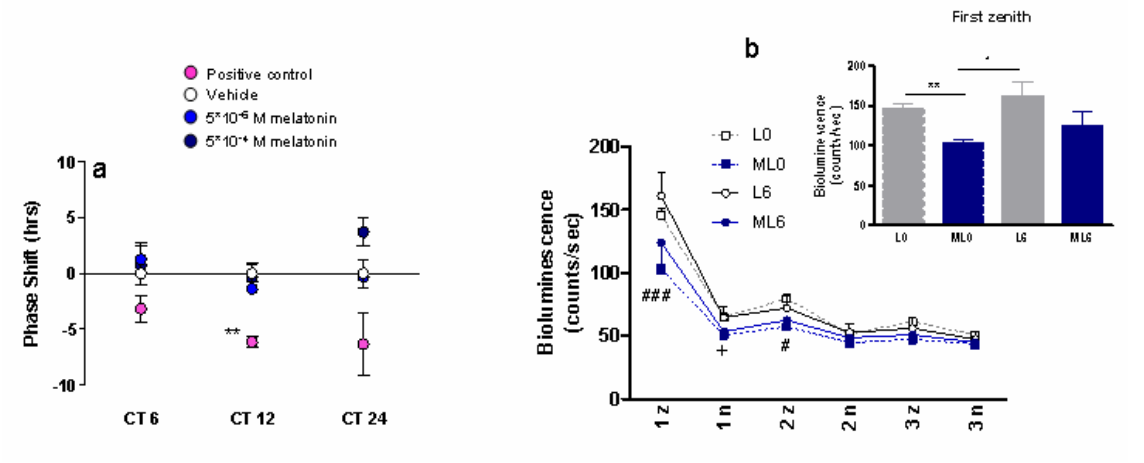

Fig. 3. Melatonin does not phase shift but affects the bioluminescence emitted by fibroblasts. (**a**) Magnitude of phase shift (y axis) of A6 upon the treatments at a given time (x axis). Every point represents the mean of 3 independent treatments ± S.E. ** $P < 0.01$. (**b**) Bioluminescence emitted by fibroblasts incubated with medium containing luciferine (L0), luciferine and melatonin $5*10^{-4}$ M (ML0), or 6 days at 37℃ and 7.5% CO_2 incubated medium containing luciferine (L6) and luciferine and melatonin $5*10^{-4}$ M (ML6). z = zenith; n = nadir. Data are plotted as mean of 3 independent measurements ± SE. # $P < 0.05$; ### $P < 0.001$. + $P < 0.05$. (**Inset**) Light emission at the first zenith. Data are plotted as mean ± SE. Unpaired t-test: ** $P < 0.01$; * $P < 0.05$.

To further confirm that melatonin does not interact with luciferine we estimated the effects of melatonin $5*10^{-4}$ M or V upon solutions of ATP at a variety of concentrations (ranged from 0.1 to 5 μM). Luciferase and luciferine were added to the ATP standard samples. The chemoluminescence emitted by the reaction seem to not differ in the presence or absence of melatonin (**Suppl. Fig. 1**). These data demonstrate that melatonin at a concentration of

$5*10^{-4}$ M did not exhibit an interaction with luciferine, but interfered with the amplitude of circadian rhythms peripheral oscillators.

The effects of melatonin on the amplitude and synchronization of peripheral oscillators reported in this study can be caused by several factors: a block of the coherence of cellular circadian rhythms due to a counteraction of the synchronization pathway by inhibiting cAMP and PKC-dependent Per1 and c-Fos expression[9] by activation of Gαi (inhibitor of cAMP signal transduction cascade) (for review see[10]), a reduction of the amplitude of the rhythm in every single oscillator, a decreasing of the protein expression, e.g. Bmal1 and luciferase, due for example to the interference of melatonin on the mRNA stability, or a combination of the two hypothesis.

Melatonin was shown to possess synchronizing properties on rat SCN slices[6], rodent behavior[5] and on the circadian rhythm of some totally blind people [11-12]. The direct effects of melatonin in animals and humans are not easy to unravel due to the complexity of the systems; hence melatonin may act directly and/or indirectly on the master clock and on neuronal oscillators. Here we describe that in a simple and very controlled system melatonin failed to directly entrain non-neuronal peripheral oscillators such as human skin fibroblasts and impaired their circadian rhythms amplitude. However, the present study cannot exclude that this effect may be relevant for peripheral oscillators synchronization in complex systems, since the decreasing of the circadian amplitude may render the cellular rhythms more tensile, increasing their sensitivity to other synchronizing signals[13]; in this case melatonin may exert an indirect action as peripheral organs synchronizator.

Acknowledgements

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Supplementary information

Supplementary methods

Subject. For this study two healthy subjects (S1 female 27 years old; S2 male, 65 years old) were recruited to donate skin biopsies. Prior ethical consent for the use of human skin biopsies was given by the Ethical Committee of Basel, Switzerland, informed consent was obtained from the subjects participating to the study.

Skin biopsies sampling, fibroblasts culture, viral infection. Three cylindrical cutaneous biopsies were taken from the buttock of the subjects. Biopsies were cultivated separately and fibroblasts were isolated by 4-hrs digestion of the tissues in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA)/2 mM L-glutamine (Invitrogen, USA)/100 U/l penicillin/100 μg/l streptomycin (Invitrogen, USA), hereafter designated as DMEMc, supplemented with 20 % Foetal bovine serum (FBS; Sigma-Aldrich, USA)/3.5 WU/ml Liberase Blendzyme 3 (Roche Applied Science, USA). After digestion biopsies and fibroblasts were cultivated in DMEMc supplemented with 20 % FBS. One separate plate of fibroblasts from each biopsy was infected using *Bmal1*::luciferase lentivirus as described previously [1]. Infected cells were selected via resistance to DMEMc/20 % FBS/5 μg/ml puromycin (Sigma-Aldrich, USA). After amplification fibroblasts were synchronized and circadian rhythm was measured as described below.

Polymerase chain reaction (PCR). The expression of transmembrane melatonin receptor hMTNR1A and hMNTR1B was evaluated using PCR protocol. Total RNA was extracted from human fibroblasts using RNeasy Mini Kit (Qiagen, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (Amersham, USA) and 0.5 μg of Oligo$(dT)_{15}$ Primer (Promega, Italia) following the manufacturer's instruction. PCR amplification was carried out using a 25 μl reaction mixture containing: cDNA, 2 μl in 10× buffer, 0.2 mM dNTPs, 0.1 U/μl U of Taq DNA Polymerase (Promega, Italia), 40 pmol of hMTNR1A (5‘-agtcagtgggttcctgatg-3’; 5’-cattgaggcagctgttgaaatacg-3’), 40 pmol of hMTNR1B (5’-caacttctttgtggggtccctg-3’; 5’-gatagggaggaggaagtggatgac-3’), 1 pmol of hCDK4 (frw5’-ATCCCAATGTTGTCCGGCTG-3’; rev5’-TGATCTCCCGGTCAGTTCGG-3’). A sample containing all the reaction reagents except cDNA was used as PCR-negative control in any amplification. Mixtures were incubated at 95 ℃ for 11 min, for 35 cycles of amp lification (1 min at 95 ℃, 1 min at 57 ℃

and 2 min at 72 ℃), and then at 72 ℃ for 5 min, u sing a Robocycler Gradient 40 (Stratagen, USA). PCR products were electrophorized in 1.5 % agarose gel together with a DNA ladder of 100 base pairs (Promega, Italia) and visualized by ethidium bromide.

Western blot. Human fibroblasts were homogenized by sonication in lysis buffer (150 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 2mM EDTA supplemented with phosphatase and protease inhibitors (Roche Applied Science, USA)) and centrifuged 1000 g for 5 minutes at 4℃. The supernatant was used fo r Western blotting. Ten micrograms of total proteins were dissolved in a loading buffer (0.1 M Tris–HCl buffer, pH 6.8, containing 0.2 M DTT, 4 % SDS, 20 % glycerol, and 0.1 % bromophenol blue) and separated by 10 % SDS-PAGE, and electrophoretically transferred to a PVDF membrane (Amersham, Germany). Equal protein loading was confirmed by Ponceau Red staining (Sigma, Germany). Membranes were saturated with 5 % non-fat dry milk for 1 h and incubated with the primary antibodies goat anti-MTNR1A (Santa Cruz, USA) or goat anti-MTNR1B (Santa Cruz, USA), overnight at 4℃. PVDF membranes were i ncubated with rabbit anti-goat biotin conjugated IgG (DAKO, USA) 1h at room temperature and then with Streptravidin horseradish peroxidase conjugated (Sigma, USA) for another hour at room temperature. As internal control, the membranes were stained for hGAPDH using a rabbit anti-GAPDH antibody (AbCam) 1 h at room temperature and then subsequently an antibody anti-rabbit horseradish peroxidase conjugated (Sigma, USA) 1 h at room temperature. Blots were developed with an enhanced chemiluminescent (ECL) assay (Amersham, Germany).

Stripped FBS. To remove steroid hormones and other organic substances from a certain volume of FBS, the same volume of a solution of 0.25 % activated charcoal (Sigma-Aldrich, USA) and 0.0025 % dextran T-70 (Sigma-Aldrich, USA), 0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM Hepes, pH 7.4 was incubated overnight. After centrifugation at 500 g 10 minutes and removal of the supernatant the serum to be stripped was added to the charcoal pellet and the solution was incubated 12 hours at 4℃ in agitation. The stripped serum was then centrifuged 500 g 10 minutes and the supernatant was filtered through 0.22 μm diameters pores.

Pre-synchronization and measurement of circadian rhythms. Circadian rhythms of infected fibroblasts were pre-synchronized either via dexamethasone treatment or via one wash using phosphate buffer solution (PBS) and replacement with the counting medium. For dexamethasone synchronization 100 nM dexamethasone (Sigma, USA) were added to

DMEMc/20 % FBS and cells were incubated for 15 min with this medium. After 3 washes using PBS counting medium containing DMEMc without phenol red/10 % stripped FBS/0.1 mM luciferine (Molecular Probe, USA) was replaced to the cells and light output was measured (3 measurements per condition) in Lumicycle (Actimetrics, USA). For each luciferase measurement, circadian parameters such as amplitude of oscillations and phase were calculated using the software Lumicycle Analysis (Actimetrics, USA).

Synchronization experiment. To the cover of the dishes a small hole of the dimension of 23–25 gauge, 5/8" needle was digged to allow the treatments to occur without perturbing CO_2 levels, temperature or light to the dishes. Cells were pre-synchronized using dexamethasone incubation for 15 minutes. Circadian time (CT) 0 corresponds to the peak phase of *hBmal1::luciferase* rhythms. Starting from the fourth cycle, cells were treated with 100 µl at CT 6, CT 12 and CT 24 (Fig. 1 inset) with positive control (dexamethasone 100 nM), vehicle (DMEMc without phenol red), melatonin at different final concentrations ($2*10^{-11}$ M, $2*10^{-8}$ M, 10^{-6} M, $5*10^{-6}$ M, $5*10^{-4}$ M). Measurements were conducted for a total of 8 days. The concentrations of melatonin used in this experiment were chosen on the basis of the melatonin binding curve[2] to range from concentrations were no receptor bound melatonin to concentrations were the 100 % of the receptors bound by melatonin. The synchronization was quantified as the percentage of the amplitude of the cycle after the treatments (A6) compared to the cycle before the treatments (A3) versus vehicle (100%). Phase shift was determined as the difference between the phase of the peak after and before the treatment versus vehicle phase.

ATP standard curve. In a white 96-wells plate an ATP standard curve was performed in duplicate using the following concentrations: 5 µM, 4 µM, 3 µM, 2.5 µM, 2 µM, 1 µM, 0.5 µM, 0.1 µM. Luciferine and luciferase were added as it is described in ViaLight MDA Plus manual (Cambrex, USA) in the presence of melatonin $5*10^{-4}$ M or vehicle. Luminescence was measured using Fluoroscan Ascent (Thermo Fisher Scientific, USA).

Bioluminescence measurement. The counting medium DMEMc without phenol red/10 % stripped FBS/0.1 mM luciferine was supplemented with melatonin $5*10^{-4}$ M (ML6) or vehicle (L6) and was incubated at 37℃ and 7.5 % CO_2 for 6 days. Counting medium was then prepared fresh and supplemented with melatonin $5*10^{-4}$ M (ML0) or vehicle (L0). To synchronize the oscillators, cells were washed once with PBS and then the media were

added to the fibroblasts. Measurements of the bioluminescence were started in the Lumicycle. The quantification of bioluminescence was performed with the software Lumicycle Analysis.

Data analyses. Statistical analyses were done using two-way ANOVA, Bonferroni post hoc test to compare replicate means by row, one-way ANOVA, Bonferroni post hoc test to compare all pairs of columns, unpaired t-test. $\alpha = 0.05$ for all the analyses. Results are expressed as mean ± standard error.

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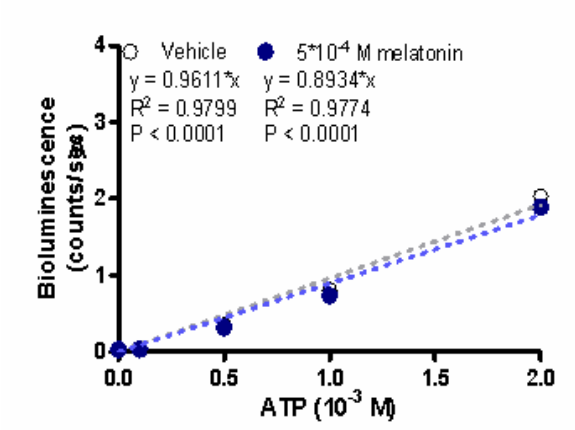
Supplementary figure



Suppl. Fig. 1. Melatonin does not interact with luciferine**.** Melatonin ($5*10^{-4}$ M; blue filled dots, blue line) seems to not influence the light emitted by the reaction of ATP, luciferine and luciferase compared to vehicle treated reactions (empty dots, grey line). Data are plotted as mean of 2 independent measurements ± SE.

5. CONCLUSIONS

Circadian rhythms control many human physiological processes, such as hepatic, metabolic and circulatory system function [1-3], mood and alertness (for review see [4]). An increasing number of evidences highlight the role of circadian rhythms disruption or misalignment in human pathologies; in diseases like depression, Alzheimer's disease but also ageing circadian disregulations are very well described [5-7] and circadian re-alignment and chronotherapies can be successful treatments [8] (for a review see [9-10]). The comprehension of the mechanisms and function of circadian rhythms can be of crucial importance for the circadian manipulation and treatment of several pathologies. However, human studies of circadian rhythms have several disadvantages: they are very labour intensive and extremely expensive, the number of subjects is limited, exclusion criteria are limiting factors (age, gender, life-style habits, etc...) and some patients have difficulties to participate due to the low compliance caused by the use of relatively invading devices. The aim of the present thesis was to validate human skin fibroblasts as a model to study human circadian rhythms *in vitro*, to use this model to investigating age-related changes and to explore the role of melatonin in the human circadian system.

In our experiments we demonstrated that human skin fibroblasts are able to parallel characteristics of human circadian physiology. Previous studies in animals suggested that cell lines, cells of the master clock suprachiasmatic nucleus (SCN) or tail fibroblasts, isolated from animals possessed a similar period length as the animal locomotor activity period [11-12]. However, the present study gives the first direct evidence about the existence of a correlation between *in vivo* period length and *in vitro* human fibroblasts period length. Moreover, fibroblasts are insensible to the effects of light as masking agent, effects that are still present in the *in vivo* protocols, suggesting that these cells are a very reliable model for the study of human circadian properties. Our results suggest that human fibroblasts may be a predictor of more than one human physiological parameter. Prior studies evidenced that extreme chronotype scores correlated with human skin fibroblasts period length [13], similarly to the correlation between chronotype and *in vivo* period length [14]. Here we show that, although the number of subjects was small, fibroblasts period tended to correlate with *in vivo* phase of entrainment between melatonin onset and sleep and wake time during the first night of constant routine. Our data demonstrate the potency

of human skin fibroblasts as cellular model to unravel the mechanisms of human circadian rhythms.

On the basis of these results, the circadian parameters of skin fibroblasts, isolated from a group of young and a group of older subjects, were characterized, to investigate the molecular mechanisms by which human ageing affects circadian clocks. During ageing the amplitude of circadian rhythms decreases, as it is reported by human melatonin or activity cycles [15] (for a review see [16]). Although a difference in chronotype between young and older biopsies donors was found, confirming published data [15], no correlation between the chronotype and the period length was detected; this apparent contrast with previous results [13] can be explained by the absence of extreme chronotypes in our study. Results about circadian parameters indicate that the molecular components of peripheral oscillators are not affected by ageing: period length, amplitude and phase are not different between the cells from the two groups of subjects. However, measuring fibroblast circadian rhythms in the presence of sera from older subjects, a shorter period and earlier phase were observed compared to the same cell lines measured in the presence of sera from young donors. Interestingly, a shorter period [17] and earlier phase [18] were also observed in studies on aged animals, although in humans only an earlier phase was reported [19]. A hypothesis that might explain these discrepancies is that circulating factors present in aged people are not able to entrain the period of the clock pacemaker, although peripheral oscillators, that are sensitive to many signals for their nature of peripheral oscillators, may respond to that substance by period shortening and phase advancing; this process could result in an earlier phase of human circadian parameters, with no effects on the period. Results about the nature of the substance responsible for the circadian changes observed in our experiments revealed that the only effect of heat-inactivation was the lengthening of the period of cells treated with sera from older donors; we could conclude that probably the circadian disturbances that affect older persons are caused by one or more heat-sensitive substances present in the blood of older subjects.

The importance of fibroblasts as cellular model in circadian rhythm studies is highlighted in functional studies. Melatonin is able to regulate SCN firing rate and entrain locomotor activity of animals and humans [20-22]. The mechanisms that underlie the entrainment of rodent and human activity may be different due to the opposite nature of the models: rodents are nocturnal and produce melatonin during their active period,

whereas humans are diurnal and produce melatonin during their rest period. Moreover, in humans melatonin was shown to facilitate sleep onset, probably by influencing the body temperature [23-24], suggesting a circadian role of melatonin prevalently in the central nervous system; however, the presence of the hormone in all the biological fluids may underlie other functions of the hormone on peripheral organs. Our studies on human skin fibroblasts revealed that melatonin was not able to re-synchronize damping fibroblasts; however, an effect in decreasing the circadian output was observed after melatonin treatment. This amplitude decrease is not caused by the reaction of melatonin with luciferine, the fibroblast output substrate. Our experiments indicate that melatonin does not directly synchronize the peripheral oscillators; however, our data cannot exclude that the effect of melatonin on the amplitude of fibroblasts may render their circadian rhythms more sensitive to other stimuli, favouring the entrainment of the clocks by other signals present in the periphery [13].

Taken together our results highlight human skin fibroblasts as important tool for human circadian rhythms studies. Fibroblast circadian rhythms parallel the donor circadian parameter, such as period length, without the problems related to *in vivo* studies. Skin cells revealed that the molecular components of every oscillator are unchanged during ageing, but probably one or more heat-sensitive substances are the factors determining the age-related circadian impairments. Moreover, fibroblasts could exclude a direct synchronizing role of melatonin in peripheral oscillators. Fibroblasts are easily isolated from a punch biopsy, a cheap, relatively invading and not time consuming technique. Many kinds of persons may be considered qualified to donate biopsies, including people suffering from physical or psychiatric diseases, subjects difficult to recruit for *in vivo* protocols. Genetic manipulation on fibroblasts is easy to perform. Moreover, fibroblasts result to be a simple model, where many variables may be easily controlled and tested. In conclusion, studies on human skin fibroblasts may be preparatory or complementary to *in vivo* studies and in some cases they may completely substitute human protocols.

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ABBREVIATIONS

AA	Arachidonic acid
AA-NAT	Arylalkylamine N-acetyl transferase
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
AD	Alzheimer’s disease
ASPD	Advanced sleep phase disorders
ATP	Adenosine-5'-triphosphate
AVP	Arginine vasopressin
BD	Bipolar disorder
BKCa	Calcium-activated potassium channels
Bmal1	Brain and muscle ARNT-like protein 1
CA	*Cornu Ammonis* of the hippocampus
cAMP	Cyclic adenosine monophosphate
CBT	Core body temperature
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CK1	Cyclin kinase 1
Cl^-	Chloride ion
CLOCK	Circadian locomoter output cycles protein kaput
CO_2	Carbon dioxide
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
CRY	Cryptochrome
CT	Circadian time
CYP	Cytochrome P 450
DBT	Double-time
DD	Dark/dark
DLMO	Dim-light melatonin onset
DMSO	Dimethyl sulfoxide
DSPD	Delayed sleep phase disorders
EEG	Electroencephalography
ER	Estrogen-receptor

ERK	Extracellular signal-regulated kinases
FEO	Food entrainable oscillator
FRH	FRQ-interacting RNA helicase
FRQ	Frequency
FSH	Follicle-stimulating hormone
FWD-1	F-box/WD-40 repeat-containing protein-1
GABA	γ-aminobutyric acid
GH	Growth hormone
GLUT	Glucose transporter
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotropin
$HCO3^-$	Hydrogencarbonate ion
HPA	Hypothalamic-adrenal-pituitary
HPG	Hypothalamic–pituitary–gonadal
IEG	Immediate-early genes
IFN	Interferone
IGF	Insulin growth factor
IL	Interleukin
ISWR	Irregular sleep-wake rhythm
JNK	Jun NH2-terminal kinases
KIR3	Inwardly rectifying potassium channel
LD	Light/dark
LH	Luteinizing hormone
LTP	Long-term potentiation
MAPK	Mitogene-activated protein-kinase
MDD	Major depressive disorder
MEK	Erk kinase
MNC	Mononuclear
mRNA	Messenger ribonucleic acid
MT	Melatonin receptor
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NK	Natural killer cells

NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
Npas2	Neuronal PAS domain-containing protein 2
NREM	Non rapid eye movement
NSAIDs	Nonsteroidal anti-inflammatory drugs
PACAP	Pituitary adelylate cyclase–activating polypeptide
PAS	PER-ARNT-SIM domain
PDE	Phosphodiesterase
Per	Period
PGO	Ponto-geniculo-occipital
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phopsholipase C
PMA	Phorbol-12-Myristate-13-Acetate
PMN	Polymorphonuclear
PRC	Phase response curve
PRL	Prolactin
PT	Pars tuberalis
PVN	Paraventricular nucleus
REM	Rapid eye movement
RHT	Retina-hypotalamic tract
RPE	Retinal pigment epithelium
RZR/ROR α	Potential retinoic-acid binding receptor/RAR-related orphan receptor
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nucleus
SIRT	Sirtuin
SKCa	Small conductance channels
SMOC	Second messenger-operated Ca2+ channel
SS	Somatostatin
Th	T-helper lymphocyte
TIEG1	Transforming growth factor ß-inducible early gene 1
TNF	Tumor necrosis factor
VDCC	Voltage-dependent Ca^{2+} channel
VDUP1	Vitamin D3 up-regulated protein 1

VIP	Vasoactive intestinal peptide
WC	White collar
WCC	White collar complex
ZT	Zeitgeber time

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Education

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"Studying human circadian behavior using peripheral cells" **Pagani L**, Eckert A, Brown SA – in press
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Travel fellowships/Honors

Trainee travel award from the Awards Committee of the 10th Gordon Research Conference on Pineal Cell Biology – Mechanisms of circadian rhythmicity and melatonin action - Pineal Gland Gordon Conference 2008 April 20th – 25th Il Ciocco, Barga, Italy

Travel award for free accommodation XI European Biological Rhythms Society Congress 2009 August 22nd – 28th Strasbourg, France

Travel fellowship from the “Vizerektorat Forschung & Nachwuchsförderung” for the Chronobiology Gordon Conference 2009 July 19th – 24th Salve Regina University, Newport, Rhode Island, USA

Trainee travel award from the Awards Committee of the Gordon Research Conference on Pineal Cell Biology – 2010 February 7th – 12th Hotel Galvez, Galveston, Texas, USA

Invitation to free membership of the Society for Research on Biological Rhythms (SRBR) 2008

Invited oral presentations

27th Annual Meeting of the Swiss Society of Biological Psychiatry - «Sleep in Psychiatry- State of the Art» - 15th March 2007, Hotel Hilton - Basel, Switzerland - *The Effects of Age upon Human Molecular Circadian Rhythms* - Abs book O-7

Society for Research on Biological Rhythms Meeting - 20th May 2008 – Sandestin, Florida – *Age and peripheral human molecular circadian rhythms* – Abs book 50 pg. 86

SLTBR Meeting 2009 June 24th – 27th Berlin, Germany - *A molecular look at chronotype and aging*

XI European Biological Rhythms Society Congress 2009 August 22nd – 28th Strasbourg, France - *Human molecular circadian rhythms and ageing* - Abs book pg. 97

6. APPENDICES

6.1 APPENDIX 1

STUDYING HUMAN CIRCADIAN BEHAVIOR USING PERIPHERAL CELLS

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ABSTRACT

Complex behaviors are the product of intercellular signalling events, but their intracellular effectors are present in most cell types. The best-studied example of such architecture is the circadian clock, which directs all diurnal behaviour and physiology, and whose central mechanism is present in most body cells. We present below a method to look at its properties via transcriptional reporters virally delivered to primary cells. By studying primary fibroblasts cultivated from skin biopsies in different human subjects, we have been able to analyze the molecular underpinnings of variance in human daily behaviour. Similar methodologies could be applied to other signalling pathways.

INTRODUCTION

Human behavior is influenced by many genetic and environmental factors, and is the product of complex inter-neuron interactions; it is therefore often difficult to study by reductionist approaches. Nevertheless, it is increasingly clear that fundamental intracellular pathways are critical to behavior, and that these same pathways are conserved in non-neuronal cell types. The premise of this chapter is that inter-individual genetic differences that create changes in behaviour do so at least in part by modifying conserved intracellular signalling pathways, and that these changes are paralleled by changes in non-neuronal cell types. Hence, they can be studied in peripheral cells obtained from human subjects.

The most clearly illustrated example of this approach can be found in recent studies of the circadian clock, which is the molecular basis for human diurnal rhythms. This clock, which has a period of about 24 hours -- "*circa diem*" -- is present in most cells of the body, and plays a key physiological role in the adaptation of living organisms to the alternation of day and night. In mammals circadian rhythms influence nearly all aspects of physiology and behaviour, including sleep-wake cycles, cardiovascular activity, endocrinology, body temperature, renal activity, physiology of the gastro-intestinal tract, and hepatic metabolism (Gachon et al., 2004). As we discuss in detail below, although specific mutations in circadian clock components have profound effects upon behaviour, these effects are paralleled by changes in circadian gene expression in easily accessible tissues such as skin fibroblasts, and hence these cells can be used as both diagnostic and analytical tools in the study of circadian behaviour.

This chapter is divided into three parts. In the first part, a general introduction to circadian clocks and their influence upon human behaviour is provided. The second part contains the protocols used by our laboratory to study circadian clock function in primary human cells. The chapter then closes with a discussion of future applications.

Basic mechanisms of the circadian clock

The idea that genes control diurnal behaviour in higher organisms was first established in the fruit fly by the demonstration that certain mutations cause altered circadian rhythms (Konopka and Benzer, 1971). The responsible gene, *period* (*per*), was subsequently isolated (Bargiello et al., 1984) and was found to be expressed in a circadian pattern

(Hardin et al., 1990). This finding, and similar data from bacteria, fungi, and plants, has led to the general idea that periodically expressed genes constitute the physiological basis of circadian clocks in all living organisms (Dunlap, 1996; Hall and Rosbash, 1993; Takahashi, 1995). Moreover, because clocks exist in virtually all light-sensitive organisms, both multi-cellular and unicellular, it is not surprising that they are cell-autonomous. Their mechanism is based upon feedback loops of transcription, translation, and phosphorylation. In the simplest organism known to possess circadian rhythms, the cyanobacterium *Synechococcus crassa*, three genes -- *kaiA*, *kaiB*, and *kaiC* -- play essential roles in a central phosphorylation feedback loop, coupled to a transcriptional one (Ishiura et al., 1998; Nakajima et al., 2005). In *Neurospora crassa*, the hierarchy is reversed, with a transcription/translation feedback loop of Frequency and White Collar proteins (Froehlich et al., 2003) probably playing a central role that is fine-tuned by phosphorylation by casein kinases 1 and 2 (He et al., 2003). Starting with metazoans, the proteins of the clock are highly conserved in sequence and in function. The same protein families that control the circadian clock in flies control the circadian clock in mammals, and polymorphisms in the genes that encode these proteins affect daily behaviour in human beings.

Many reviews have been written about the molecular workings of the mammalian circadian oscillator (Ko and Takahashi, 2006), and we outline these mechanisms only briefly here. As in simpler organisms, the key mechanism is interconnected feedback loops of transcription and translation. It is conventional to divide clock components into a positive limb (consisting of transcriptional activators of clock genes) and a negative limb (inhibitors of these transcription factors). In mammals, the negative key components of the genetic circuitry are the four genes encoding cryptochrome 1 (*Cry1*) and cryptochrome 2 (*Cry2*) (Fig.1, green rhombus), period 1 (*Per1*) and period 2 (*Per2*) (Fig.1 violet ellipse). These genes are regulated by the two PAS domain basic helix-loop-helix transcription factors BMAL1 (Fig.1, red octagon) and CLOCK (or in some tissues NPAS2) (Fig.1, blue hexagon), the key positive components of the circadian oscillator. CLOCK and BMAL1 form a heterodimer that binds specific sequences present in *Per* and *Cry* promoters, the E-boxes, and activates the transcription of *Per* and C*ry* genes. Their gene products, PER and CRY proteins, in turn form heteropolymeric complexes of unknown stoichiometry that interact with the CLOCK-BMAL1 heterodimer, leading to a block of *Per* and *Cry* expression. As a consequence *Cry* and *Per* mRNAs and proteins decrease in concentration, and once the nuclear levels of the CRY–PER complexes are insufficient for

auto-repression, a new cycle of *Per* and *Cry* transcription can start (Albrecht and Eichele, 2003; Reppert and Weaver, 2002).

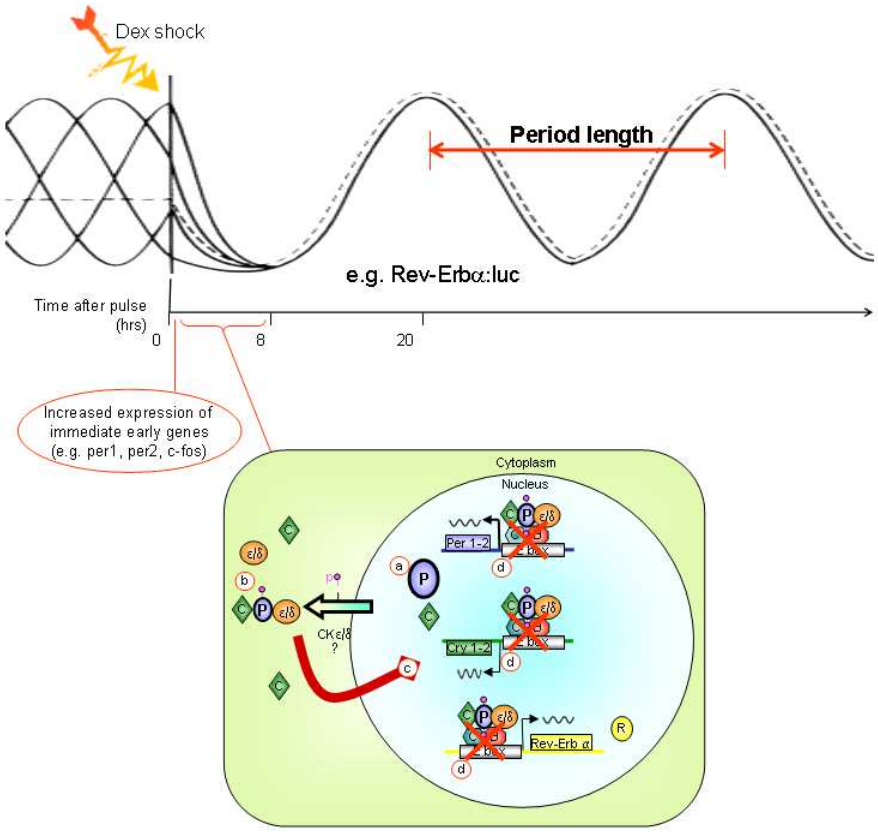

FIGURE 1. Schematic diagram of fibroblast synchronization and period measurement. In each cell on a plate of cultured cells, a separate circadian clock ticks. The heterodimer of CLOCK (C, blue hexagon) and BMAL1 (B, red octagon) activates the transcription of period genes *Per1-2* (P, violet ellipse), cryptochrome genes *Cry1-2* (C, green rhombus) and *Rev-Erbα* (R,yellow ellipse) through E-box enhancers. As the translated PER1-2 protein levels rise in the cytoplasm they multimerize with CRY1-2 protein and casein kinase ε/δ (CK ε/δ) (ε/δ, organge ellipse) and are phosphorylated (p, pink circle). The complex re-enters in the nucleus where it binds the dimer CLOCK-BMAL1, inhibiting its transcription activity. Meanwhile, REV-ERBα binds the Rev-Erb/ROR responsive elements present in the *Bmal1* promoter, repressing *Bmal1* transcription. Since each cellular mechanism is unsynchronized with that of its neighbour, the population average of clock gene expression is constant (dashed line). The addition of dexamethasone results in synchronous activation of period genes (a), whose protein products multimerize with the more abundant cryptochrome proteins CRY1-2 (b) and are translocated to the nucleus (c). These complexes then inhibit the activation of circadian genes by CLOCK and BMAL1 heterodimers (d). The resultant repression of clock genes "resets" the clockwork and synchronizes the oscillators of independent cells, allowing precise measurement of circadian period from population cultures. (Adapted from Balsalobre et al., 1998).

Additional interlocked feedback loops contribute to the robustness of this molecular clockwork circuitry. For example, the orphan nuclear receptor and repressor REV-ERBα (Fig.1, yellow ellipse) interconnects circadian transcription of the positive and negative "limbs" of the oscillator. *Rev-Erbα* transcription is activated by the CLOCK-BMAL1 complex through the binding to E-box sequences present in its promoter, resulting in a

circadian accumulation of REV-ERBα. REV-ERBα leads to periodic repression of *Bmal1* transcription. In turn, this leads to a rhythmic expression of *Bmal1* mRNA that is antiphasic to *Rev-Erbα* expression (Preitner et al., 2002). Thus, indirectly, *Bmal1* is positively regulated by PER and CRY proteins.

Posttranslational mechanisms such as protein phosphorylation also play important roles. For example, casein kinase 1ε (CK1 ε) (Fig.1, orange ellipse), initially identified as an essential Drosophila clock component (Price et al., 1998), phosphorylates PER, CRY, and BMAL1 proteins (Eide et al., 2002; Eide and Virshup, 2001; Lee et al., 2004). CK1δ (Fig.1, orange ellipse), a close paralog of CK1 ε, has also been found to be associated with PER–CRY complexes and may therefore perform a similar function as CK1 ε (Lee et al., 2001). While some phosphorylations of PER proteins stabilize it, others destabilize it, leading to a complex balance of post-transcriptional regulation that helps to determine period length (Vanselow et al., 2006).

Central and peripheral oscillators

Ablation and transplantation studies have firmly established that the suprachiasmatic nuclei (SCN) of the brain anterior hypothalamus are the site of the master circadian clock in mammals (Ralph et al., 1990). Surprisingly, recording of spontaneous action potentials from individual SCN neurons showed that the oscillatory machinery of the SCN is cell-autonomous as in simpler organisms (Welsh et al., 1995), and many laboratories therefore began looking for clock genes and other clock tissues.

It was soon noted that these “clock genes” are expressed rhythmically outside the SCN (Sun et al., 1997), and even outside the brain (Zylka et al., 1998) in many peripheral tissues. The use of transgenic *Drosophila* that express green fluorescent protein under the control of the promoter of the clock gene *period* permitted researchers to follow in real-time the oscillation of the circadian rhythm in dissected peripheral tissues such as head, thorax, and abdominal tissues, demonstrating that autonomous circadian oscillators are present throughout the body of a fly (Plautz et al., 1997). The same result was rapidly shown to be true for zebrafish and mammals, and even for immortalized fibroblasts (Balsalobre et al., 1998; Whitmore et al., 1998; Yamazaki et al., 2000).

Communication between the SCN and Periphery

The SCN uses humoral signals, neural efferents, and indirect physiological cues (neural control of body temperature and feeding time) to convey timing information to other parts of the brain and the periphery. On an anatomical level, many hypothalamic nuclei receive SCN projections that mediate the circadian control of multiple systemic cues. For example, both direct and indirect projections from the SCN make synaptic contact with CRH (corticotropin-releasing hormone) neurons of the paraventricular nucleus (PVN). These neurons in turn impose rhythmic ACTH (adrenocorticotropic hormone) release from the pituitary and subsequent circadian corticosterone secretion from the adrenal glands. The sleep/arousal, reproductive, and endocrine systems are also regulated by the SCN in part through neuroanatomic connections. In addition, there exist SCN connections to all major organs via both sympathetic and parasympathetic pathways, and recent evidence suggests a critical role of the ANS (autonomic nervous system) in synchronizing peripheral physiology (Buijs et al., 2003). Interestingly, though, a transplanted SCN encapsulated in porous plastic is still able to direct circadian locomotor activity (Silver et al., 1996), so at least this fundamental output of the circadian clock does not depend upon synaptic transmission directly from the SCN.

Hormonal signals probably also play an important role in communication between the SCN and peripheral clocks. Glucocorticoids agonists can effectively shift peripheral clock gene expression in mice (Balsalobre et al., 2000a). The precise role of other cyclically secreted hormones such as GH (growth hormone) and PRL (prolactin) in peripheral phase entrainment is yet to be examined. The SCN can also indirectly entrain peripheral oscillators by controlling daily activity/rest cycles, and consequently, feeding time (Damiola et al., 2000; Stokkan et al., 2001). Hormones and metabolites related to feeding, such as insulin and glucose, might be involved in this phase setting (Hirota et al., 2002). Rhythmic body temperature -- controlled by the SCN via the preoptic anterior hypothalamus -- is also sufficient to entrain peripheral circadian oscillators *in vivo* and *in vitro* (Brown et al., 2002). Finally, activity rhythms as well as circadian outputs from other tissues can feed back on the SCN and on other peripheral oscillators. For example, melatonin released from the pineal gland, which is itself under SCN control through autonomic pathways, functions as a circadian input signal for both the SCN and the anterior pituitary (Stehle et al., 2003).

Mutations in clock genes affect human circadian behavior

In organisms from mice to cyanobacteria, mutations in clock genes have furnished an excellent tool for studying circadian clocks. In human beings, polymorphisms located in known clock genes result in circadian pathologies. One of the most studied syndromes is familial advanced sleep-phase syndrome (FASPS). Individuals with this syndrome can wake up and go to sleep hours earlier than normal people. This phase change is believed to be related to a change in the endogenous free-running period of the human circadian oscillator. Normally around 24 hours, it has been measured to be only 20 hours in an individual from an extensively studied FASPS lineage (Jones et al., 1999). The source of the difference has been mapped to a change from serine to glycine at residue 662 of the *Per2* gene. This mutation leads to a reduction in kinase activity of CK1 ε, resulting in a shortening of period length of the circadian rhythm (Toh et al., 2001). An unlinked mutation in casein kinase 1δ also results in FASPS (Xu et al., 2005).

Other polymorphisms in clock genes have been shown to be associated with a delayed sleep phase syndrome (DSPS) (Archer et al., 2003; Ebisawa et al., 2001; Katzenberg et al., 1998; Pereira et al., 2005), though specific molecular mechanisms have not yet been identified. On a more general note, basic heritability studies of twins suggest that human daytime preference (“chronotype”) is 50% heritable (Koskenvuo et al., 2007). Studies relating chronotype to circadian clock period length suggest that morning chronotype can result from a shorter period of the endogenous circadian oscillator, and evening chronotype from a longer clock period (Duffy et al., 2001). Altogether, it appears that clock workings at a cellular and molecular level in different individuals can provide mechanistic explanations for differences observed at a behavioural level.

Interaction between the circadian clock and mood disorders

Many other diseases are also linked to sleep and circadian disturbances, For example, one striking feature of circadian rhythm sleep disorders is that they are often associated with other mood disorders. A part of this association is by definition: an established clinical symptom of diseases like major depressive disorder (MDD) and bipolar disorder (BD) is abnormal sleep/wake, appetite, and social rhythms (Boivin, 2000; Bunney and Bunney, 2000) which are also hallmarks of circadian rhythm disorders. Nevertheless, an increasing body of evidence suggests that there exists an interesting genetic basis for this correlation.

Several circadian markers show abnormalities in depression (Avery et al., 1982; Duncan, 1996; Souetre et al., 1989; Souetre et al., 1988; Szuba et al., 1997). One hypothesis for the involvement of circadian rhythm disturbances and development of depression is that, perhaps, depression may involve a weaker coupling process between internal pacemakers and involve abnormal sensitivity to environmental cues such as light (Souetre et al., 1989). This could be a result of mutant clock genes or allelic variations leading to abnormal clock cycles or altered photosensitivity. In BD, a single nucleotide polymorphism in the 3' flanking region of the Clock-gene associates with a higher recurrence rate of bipolar episodes (Benedetti et al., 2003). This mutation is specific to bipolar depression: a similar association is not found in MDD (or unipolar depression) (Bailer et al., 2005). Another mutation, this time linked to the onset of illness in BD, has been localized to the glycogen synthase kinase 3β promoter (Benedetti et al., 2005). This enzyme is the target of lithium, a common treatment for BD, and can phosphorylate the clock component REV ERBα (Yin et al., 2006).

Seasonal affective disorder (winter depression, or SAD), is a syndrome characterized by recurrent depressions that occur at the same time every year (Rosenthal et al., 1984). Depressive phases are associated with hypersomnia, overeating, and carbohydrate craving. Abnormalities in circadian rhythms in SAD include sleep disturbances (Rosenthal et al., 1984), and alteration of many circadian markers (Avery et al., 1997; Dahl et al., 1993; Lewy et al., 1998; Lewy et al., 1987; Schwartz et al., 1997). Light therapy, sleep deprivation and phase-advance treatment have been used either separately or in combination to treat SAD and depressive illness. (Lewy et al., 1998). Here, too, an understanding of the molecular workings of the circadian clock in affected individuals could furnish valuable insight into the causes of these disorders.

MEASUREMENT OF HUMAN CIRCADIAN CLOCKS

Many physiological and biochemical parameters such as body temperature, melatonin, cortisol, heart rate, and blood pressure exhibit circadian rhythms. In principle, measurement of circadian parameters such as period length (the time of one complete clock cycle) or phase (the time of an organism's clock within a given cycle) could be accomplished by measurement of any of these properties. However, endogenous circadian clocks in humans are masked by environmental factors. To dissect endogenous and exogenous components of a circadian rhythm, several protocols have been developed. These protocols include "constant routine", in which subjects remain supine under constant environmental conditions, with small isocaloric meals and short naps at regular intervals (Duffy and Dijk, 2002), and "forced desynchrony", in which subjects are in a dark-light environment so short or so long (e.g. 20h period length or 28h period length) that their endogenous clocks cannot adjust and instead run freely (Blatter and Cajochen, 2007). Both of these methods employ extensive subject observation under controlled laboratory conditions; hence, they are very expensive and labour-intensive. In addition, the significant time commitment required by subjects makes their recruitment problematic.

Reporter assays in peripheral cells

To circumvent the difficulties of direct measurement of circadian behaviour in humans, our laboratory has tried instead *ex vivo* analysis of clocks in tissues that can be obtained easily – skin, blood, or hair root cells. Evidence from rodent models suggests that clocks in peripheral tissues utilise similar or identical complements of basic clock proteins, and that mutations in clock genes that affect SCN function and locomotor behaviour also affect molecular oscillations in peripheral cells (Pando et al., 2002; Yagita et al., 2001).

To measure clock function in peripheral human cells in high-throughput fashion, we have employed lentivirally-delivered circadian luciferase reporter vectors. By virtue of their easy preparation, wide host tropism, and stable integration into host DNA, lentiviral vectors are an excellent system to deliver reporters to hard-to-transfect primary cells (Salmon and Trono, 2007). Firefly luciferase is commonly used for the analysis of promoter function in eukaryotic systems (Alam and Cook, 1990). The enzyme catalyzes the oxidative decarboxylation of beetle luciferin using O_2, Mg_2 and ATP as substrates. A photon is released at 560 nm in 90% of catalytic cycles: this light emission can be quantified in a luminometer. In mammalian cell cultures, the half-life of luciferase activity is approximately

3 hr (Thompson et al., 1991), making it a convenient choice as a circadian reporter. Studies with transgenic mice containing the luciferase gene under control of clock promoters have shown that the molecular oscillations of clock gene expression reported in this fashion accurately recapitulate circadian transcription measured by conventional means in these tissues. Hence, clock gene*::luciferase* reporter systems have been widely used to measure expression of clock genes in prokaryotes, plants, *Drosophila,* and mammals (Brandes et al., 1996; Kondo et al., 1993; Millar et al., 1995; Millar et al., 1992).

Cell type considerations

In principle, circadian reporter constructs could be introduced into any cell type. Practically, though, a limited range of primary tissues are available from human hosts. These include hair root cells, blood cells, and skin cells. Hair root cells include melanocytes and keratinocytes. Melanocytes are deep cells that seldom cling to the end of plucked hair. Since keratinocytes are present in the hair sheath, they are more easily obtained, but do not proliferate to high levels. Highly proliferative pre-keratinocytes are also present in sheath, but are more difficult to cultivate. Blood monocytes are easily cultivated but have little amplificatory potential; and B- and T-cells can be amplified, but have very weak endogenous clocks and are difficult to infect with lentiviral vectors.

Because of their ease of manipulation, cultivation, and storage, our laboratory has mostly used adult dermal fibroblasts. Individual fibroblast cells are capable of functioning as independently phased circadian oscillators that are self-sustained for many days *in vitro*. They are also sensitive to a wide range of phase-shifting stimuli, including serum shock, glucocorticoids, and even medium changes (Balsalobre et al., 2000b). From these cells, a variety of properties can be measured:

Period length: By synchronizing cultivated cells chemically (e.g. with dexamethasone) and then measuring their period under constant conditions, one can obtain a measure of the free-running period length of the circadian oscillator (τ). (Figure 1) A strong correlation has been found between *in vivo* free-running behaviour from different mouse strains and period length of primary adult dermal fibroblasts from mouse tail. (Brown et al., 2005). Hence, it appears possible to use peripheral oscillators as proxies for the clocks of the SCN. Recently, similar studies have been performed in humans, and fibroblasts from

humans of extreme early chronotype show period lengths on average shorter than those from subjects of extreme late chronotype (Brown et al., 2008).

Phase response: To entrain to the daily light/dark cycle and respond optimally to changes in it, the circadian oscillator responds differently to light at different phases of its cycle. (Indeed, at some times of day – the so-called “dead zone” – the oscillator is completely insensitive to light.) This differential effect is most easily visualized as a phase-response curve (PRC), which plots the change in phase of the circadian clock as a function of the time that a stimulus is given. Under normal circumstances, fibroblasts do not respond to light. Nevertheless, the same signalling pathways that light uses to communicate to the clock in other cells – cAMP and CREB – are present. Thus, one can measure a PRC in response to an activator of cAMP (forskolin, which increases adenyl cyclise activity). Interestingly, the shape of the curve so obtained resembles the PRC of the human light response *in vivo* (Brown et al., 2008; Khalsa et al., 2003). (Figure 2) By reintroducing a photopigment into fibroblast cells, one can even measure the sensitivity of the fibroblast clock directly to light (Pulivarthy et al., 2007).

Phase: After a few days of cultivation, fibroblast cells lose synchrony and phase is randomly distributed (Welsh et al., 2004). Since the methods described here involve amplification of cells for several days, timing information is therefore lost. For all experiments described here, a prior resynchronization, usually with dexamethasone, is employed. Absolute phase information – i.e. the time at which the biopsy was taken, according to the biological clock – is thereby lost, but one can still measure the phase angle of normal entrainment for fibroblasts from any subject. In this case, fibroblasts are incubated in a shallow circadian temperature gradient (usually 12 hours at 34°C and 12 hours at 37°C) to mimic the daily entraining signals of the SCN. Cells will now adopt a period of 24 hours, but the peak of reporter gene expression within this 24-hour cycle will vary according to the period and amplitude of the underlying circadian oscillator (Brown et al., 2008). For example, the phase of fibroblasts carrying the FASPS mutation in *Per2* have a phase of bioluminescence six hours earlier than otherwise isogenic wildtype fibroblasts (Vanselow et al., 2006) in this protocol.

Amplitude: Because the magnitude of the reporter signals measured here is dependent upon the titer of virus used, amplitude is a property that cannot be directly measured at this time. It is possible, however, to cultivate identical plates of fibroblasts from the same

subject, synchronize circadian oscillations with dexamethasone, and then harvest plates sequentially over a period of 24 hours. From these cells, RNA or protein can be prepared to give a direct measure of the circadian amplitude of any gene or protein.

Figure 2

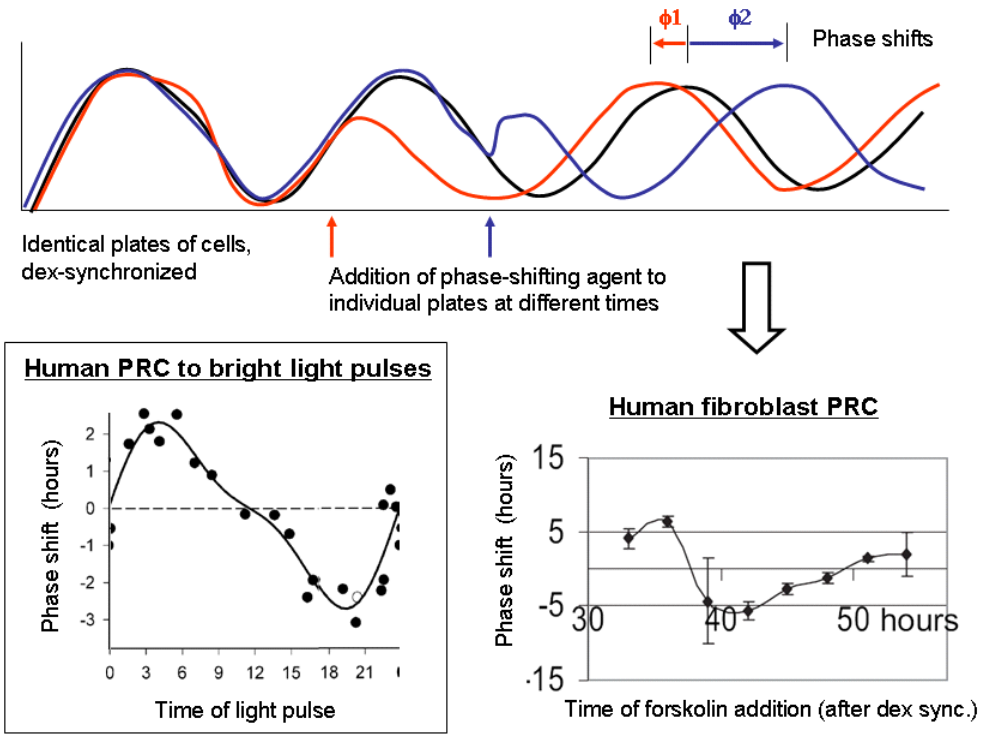

FIGURE 2. Diagram of a phase-shifting experiment. Identical plates of cells are synchronized with dexamethasone. After one day, a phase-shifting agent (in this case, forskolin) is added to each plate at a different time of day. The phase of the next circadian cycle is measured with respect to a control plate to which vehicle alone has been added. After all plates have been measured, the data can be plotted as a PRC (phase response curve) in which the magnitude of phase shift is shown for each addition time. The shape of this curve is very similar to that of a PRC of human physiology in response to bright light pulses (inset). (Adapted from Khalsa et al., 2003 and Brown et al., 2008).

PROTOCOLS

Overview of requirements

These protocols can be conducted by any laboratory comfortable with mammalian cell culture. Some special permissions, as well as the collaboration of a medical doctor, are usually required. Moreover, a real-time luminometry device capable of measuring bioluminescence in constant-temperature cell culture conditions is necessary to visualize results. Several commercial models exist (e.g. from Actimetrics, Hamamatsu, or Perkin-Elmer). Home-made devices are also feasible, and work equally well.

Ethical considerations

Collection of human fibroblasts by dermal punch biopsy is considered an invasive procedure in most countries, and therefore requires a medical doctor and prior approval of the Ethical Committee or Institute Review Board for the hospital or university in question. Typically, this permission is easily obtained for adults, but not necessarily for children.

Use of viruses

The self-inactivating lentiviral vectors used here are considered to be Biohazard Level II, which typically requires institutional notification and a separate cell culture facility. This classification applies only to the viral supernatants. Cell lines, once infected with virus, are considered to be only Biohazard Level I and may be cultivated normally.

Storage of samples

Fibroblast cells may be stored cryogenically as for any other immortalized cell line. We find that a storage medium consisting of 50% fetal bovine serum (FBS)/–Dulbecco's Modified Eagle's Medium supplemented with 4500mg/l glucose (DMEM)/7%DMSO works optimally. Viral supernatant at neutral pH may be stored indefinitely at -70℃, but has a half life of only a few hours at 37℃ or room temperature, and a week at 4℃ or on ice. Tissue biopsies may be stored in DMEM/50%FBS on ice for up to five days prior to fibroblast cultivation.

Producing the reporter virus

The circadian measurement system described here relies upon lentiviral delivery of a bioluminescent reporter construct. We have equally used adenoviruses and adeno-

associated vectors, but favour the lentivirus for its stable integration into the host genome, allowing permanent storage of infected cells as stable lines. Since lentiviruses are amphotropic vectors (i.e. they infect most mammalian cell types including human ones), all production systems employ some strategy to prevent the generation of actively replicating virus and thereby minimize danger to the researcher. Our vectors are a so-called “second generation” system obtained from the laboratory of Didier Trono (http://tronolab.epfl.ch/). One plasmid containing the vector itself is the only genetic material transferred to the target cells. It typically comprises the transgene cassette flanked by cis-acting elements necessary for its transcription, encapsidation, and integration. A second plasmid supplies all the genes necessary for packaging the virus, and a third supplies the coat protein, in our case the VSV-G coat protein for maximum host spectrum. All of these plasmids are cotransfected into human embryonic kidney cells immortalized by large T antigen (293T cells). Note that all human embryonic kidney (HEK) cells are not 293T cells, but can be immortalized by other means. The viral production plasmids contain an SV40 origin of replication, and will therefore amplify to high levels in 293T cells (but not in other HEK cells such as 293E) after transfection.

Calcium phosphate transfection in 293T cells: Typically, the transient transfection for viral production is done using calcium phosphate. In these cells, it is as efficient as lipophilic reagents, and certainly much cheaper. The procedure of virus production is 5 days long. The first day, amplified 293T cells must be at about 30-50% confluence in 10 cm Petri plates with 8 ml of medium. This medium is DMEM, pennicillin-streptomycin-glutamine solution as recommended by the manufacturer, and 10% FBS. For each ten-centimeter dish, the following quantities of plasmids are transfected: 15 μg of viral reporter plasmid (e.g. pBluFpuro), 10 μg of packaging plasmid (e.g. psPAX2), and 6 μg of coat plasmid (e.g. pMD2G). This DNA is assembled in a total volume of 400μl weakly buffered water (2mM HEPES pH 7.05). 100 μl of 2.5M $CaCl_2$, tissue culture grade, filter-sterilized, is then added, and the mixture is allowed to equilibrate to room temperature. A second tube containing 500 μl of a solution of 2x HeBS (NaCl 0.283M, HEPES 0.023M, $Na_2HPO_4$1.5mM, pH 7.05) is also equilibrated to room temperature. The pH of HeBS is fundamental for high transfection efficiency: pH 7.05 is ideal, and pH outside the range 7.0-7.1 will result in significantly reduced efficiency.

The plasmid and $CaCl_2$ mix is dropwise added to the 2xHeBS under agitation. The combined solution is vortexed for 5 seconds, incubated for 30 minutes at room temperature, and added dropwise to 293T cells. The medium is mixed by rocking the plate back and forth, and cells are incubated overnight at 37℃, 5-7% CO_2. The day after the transfection, 293T cells are washed with 5 ml of 1x PBS and then 10 ml of fresh medium enriched with 20mM Hepes 7.8 is added to the cells to stabilize the pH-labile virus. Over the next two days, virus will be released into the culture medium. The following day the virus-containing medium is harvested and stored on ice, and replaced with 10 ml of fresh medium enriched as before. The following day medium is harvested again from the cells. 293T plates are now discarded. Virus-containing medium is pooled and spun at 2G for 5 minutes to pellet debris, and then filtered through 0.4 μm filters to remove residual 293T cells.

Virus can be concentrated through ultracentrifugation at 80,000 G 4℃ for 90 minutes, ideally in conical-bottom tubes. The resulting very small, compact, whitish-translucent virus pellet is resuspended overnight by medium-speed vortexing at 4℃. The virus is stored at –80℃. A concentration of virus between 7 and 10 x i s ideal for a good cell infection. Higher titers can be toxic for primary cell cultures.

Optimising virus production: Typically, we perform two tests to quantify virus production. In the first, we routinely transfect a plate of GFP-expressing vector in a duplicate plate in parallel with our larger preparations of virus. After two days, the efficiency of transient transfection can be analyzed directly by fluorescence microscopy. About 50% is expected. The same plate can be left to produce virus, which is concentrated as usual. One aliquot of concentrated virus is then rediluted with medium to its original volume. Both of these aliquots are tested by infection of 293T cells (see below). 100% infection is expected in both cases. If desired, exact titers can be determined by serial dilution.

Processing of skin biopsies

The punch biopsy is a fast, easy, inexpensive method to produce a cylinder of tissue from the skin surface to the underlying subcutaneous fat. A two-millimiter round dermal punch is sufficient, and disposable needles for this purpose are commercially available. Typically, the punch biopsy is taken from the buttocks or upper arm of a subject. The area chosen for the sampling is an area that normally is not exposed to sunlight. The reasons for this

choice are a reduced risk of mutagenesis caused by light rays to healing wounds and reduced aesthetical problems caused by the healing lesion for a few weeks after the sampling. No permanent scarring results.

Preparation of the skin: The skin should be prepared with chlorhexidine (Hibiclens), or povidone iodine (Betadine, Isodine), or alcohol. This is performed as a clean procedure, and full sterile technique with sterile drapes is unnecessary. Local anesthesia with lidocaine can be used at patient request. One percent lidocaine with epinephrine is injected via a 30 gauge needle and Luer-Lok syringe, about 50µl on each side of the planned biopsy site if desired.

Punch: With the skin held taut at right angles to skin tension lines, the cutting edge of the biopsy needle is pressed down firmly, and simultaneously twisted one way and then the other in quick succession. As the punch traverses the dermis, resistance is lost, and there is a slight “give”. Typically, the punch will remain in the needle as it is removed. Sterile tweezers or a 26 g needle should be available to remove it. Occasionally, the biopsy may remain in the wound, and the same removal procedure applies. Bleeding is resolved with pressure, and no sutures are necessary for a 2mm biopsy punch. A bandage is applied for a few days after the procedure. For every biopsy a collection tube filled with medium must be prepared and kept on ice. The medium used to collect the biopsy is DMEM containing 50% FBS, plus a suitable concentration of penicillin, streptomycin, and fungicide such as Amphotericin B as recommended by the manufacturer. Tubes should be almost filled to facilitate immersion of biopsy, which adheres easily to dry surfaces. Biopsies in this ice cold solution can be kept for up to 5 days. Shipping of the biopsy can occur at this stage.

Biopsy processing: Biopsies are transferred in a 3.5 cm Petri dish for processing. Pour 2 ml of DMEM, 20%FBS, Amphotericin B, 200 µl of liberase in the dish with the biopsy. Liberase (Roche) is an enzyme blend of collagenase isoforms I and II from clostridium histoliticum and thermolysin from bacillus thermoproteolyticus; its action is to destroy partially the extracellular matrix, to allow the growth of fibroblasts outside the biopsy. Incubate the biopsy with the liberase-containing medium at 37℃, 5-7% CO_2 for 4-10 hours. At the end of digestion multiple clumps containing tens to hundreds of cells are present, though the original biopsy has not entirely dissolved. The biopsy is transferred to 10 ml of PBS 1x and centrifuged for 5 minutes at 200 g. The biopsy pellet is resuspended

in 200 µl of DMEM, 20% FBS, penicillin-streptomycin+Amphotericin B and transferred back in the middle of the surface of the 3.5 cm Petri dish. To maintain the biopsy attached to the bottom of the plate a home-modified Millicell CM membrane disc (Millipore) from which spacer feet have been removed is placed on the biopsy. The membrane disc is covered with 1.5-1.8 ml of the same medium and incubated overnight. The following day the medium is changed. After a few days the first fibroblasts start to grow around the biopsy. After one week of cultivation of the biopsy it is not necessary to add amphotericin B to the medium. 7-10 days after the biopsy processing the biopsy is attached to the bottom of the plate and the membrane disc can be carefully removed. The biopsy requires changing of medium every 3-4 days. Length of culture before the first harvest of fibroblasts will vary with the number of viable fibroblast foci. Cells are ready to be harvested when the combined volume of their foci is about ½ the volume of the plate to which they will be amplified. Trypsinize and replate cells normally. It should be noted that biopsy cultivation can lead initially to the growth of more than one kind of cells. Keratinocytes can grow from the biopsy; however, they will be overrun by the faster-growing fibroblasts.

Splitting procedure: To split the cells medium is removed and cells are washed once with PBS1x. About 0.4 ml of trypsin is enough to detach fibroblasts that grow in a 3.5 cm Petri plate. The detachment of the cells can be followed under the microscope. The trypsin reaction should not be prolonged because human primary fibroblasts can be damaged. Normally trypsinisation of fibroblasts takes about 1 minute. After this time the plate is vigorously shaken to promote the detachment of the cells. 2 ml of serum-containing medium are then added to block the reaction of trypsin. Cells are usually split 1:2 during each amplification passage.

Freezing procedure: Trypsinise fibroblasts as described in the splitting procedure. For every plate of fibroblasts a 15 ml polypropylene tube filled with 10 ml of PBS1x is prepared. Cells are detached from the bottom of the plate and transferred in the tube. Centrifugation for 5 minutes at 200 g is necessary to pellet fibroblasts. The supernatant is removed and cells are suspended in 1.5 ml of DMEM, 50% FBS, 7% DMSO. Cells are transferred into an ice-cold cryovial. The vial is left at least 20 minutes at -20℃ and then placed at -80℃ overnight. The following day cells can be definitively stored in liquid nitrogen. Human primary fibroblasts can be stored in liquid nitrogen for years.

Thawing process: For every vial of cells to be thawed, a 15 ml polypropylene tube with 10 ml of 37℃ warmed medium must be prepared. Unfreeze fibroblasts quickly, placing 0.5 ml of 37℃ warmed medium in the cryovial and transferring the medium into the prepared tube. Repeat this operation until all the cells have been thawed and moved into the tube. Centrifuge the tube 5 minutes at 200 g to pellet the cells. Remove the supernatant and resuspend the cells in 2 ml growth medium (as above) and plate into the same size of plate as originally used for freezing.

Measurement of circadian oscillations from skin biopsies

Fibroblast infection: Even though lentivirus can infect non-replicating cells, the infection efficiency is increased when the cells are in mitosis. On the day of the infection, fibroblasts should be about 30-50 % confluent. For a 3.5 cm Petri plate it is enough to use about 750 µl of warmed concentrated virus prepared as above. To increase the transfection efficiency, 8 ug/ml protamine sulfate is added to the virus aliquot as a polycation to block non-specific virus binding to the cell surface. Fibroblasts can be incubated overnight, though the infection is complete in about 6 hours. The following day the virus-containing medium is removed from the cells. Fibroblasts are rinsed with PBS 1x and standard DMEM high-glucose medium containing 20% FBS and penicillin-streptomycin is added to the cells. Three days after the viral transfection antibiotic selection of transfected cells can be started, if desired.

Measurement of circadian period length: When transfected fibroblasts have reached 90-100% confluence the measurement of the circadian rhythm can be started. It is necessary to synchronize fibroblast rhythms. Commonly used methods are serum shock or a pulse of the glucocorticoid agonist dexamethasone. To induce synchronization with serum shock, cells are incubated with DMEM and 50% FBS for one hour. In the case of synchronization through dexamethasone, fibroblasts are treated for 15 minutes with DMEM, 20% FBS, 100 nM dexamethasone. Cells are washed with PBS 1x to remove the presence of synchronization agents and medium is replaced. The medium that is used to measure the circadian rhythm is DMEM without phenol red, 10% FBS, penicillin-streptomycin, and 0.1 mM luciferin. If evaporation is a problem, plates can be sealed with parafilm prior to placement in the measurement machine. In this case, medium should be buffered with 20mM HEPES pH 7.6. Normally, circadian oscillations can be observed for 5-6 days. (The induced circadian oscillations are diagrammed in Figure 1.)

Phase response experiments: For these experiments, duplicate plates – one for each timepoint desired in the phase response curve – are synchronized with dexamethasone as above. Plates are measured together for 1 day, and then the phase shifting agent is added at the desired time to one plate (experiment) and vehicle to the other (control). It is important that all reagents be prewarmed and that the procedure be done quickly, because temperature is also a phase shifting agent. (Figure 2)

Phase entrainment experiments: For these experiments, it is necessary to change incubator temperature between 34 and 37℃ every 12 hours for six days. (One can purchase cell culture incubators that will do this automatically.) Fibroblast cells are placed into this regime, and on the seventh day, temperature is kept constant at 37℃ and bioluminescence is measured. (Figure 3) Because the electrical noise and quantum efficiency of the photomultiplier tube is itself temperature-sensitive, the temperature of the incubator should not be varied during the day of measurement unless a special insulating measurement device is constructed.

Data analyses: To analyse the circadian rhythm of human primary fibroblasts, either commercial software (e.g. Lumicycle Analysis, from Actimetrics), or Matlab routines (available from Hanspeter Herzel, Humboldt University Berlin) can be used. Both programs begin by normalizing data to a 24-hour running average. To determine period, the programs then find the period and time constant that best fit the data to a sine wave multiplied by an exponential decay

$$C = A \sin(2\pi t/P - t_0) * e^{-t/\tau}$$

Empirically, we generally discard the first 18 hours of data because noncircadian fluctuations associated with the synchronization protocol make automatic sine fitting more problematic. To determine phase shifts during a PRC experiment, the timing of the peak of reporter expression after substance addition in experiment and control samples are manually determined and compared to each other. The phase response curve contains the magnitude of these differences on one axis, and the time of substance addition (relative to dexamethasone synchronization) on the other axis. For phase entrainment experiments, the peak of reporter gene expression is reported relative to the onset of the 34:37 temperature cycle.

Troubleshooting

Magnitude of signal: Here, problems are usually related to viral titer. We recommend routinely testing transfection efficiency and viral titer at the time of viral production by using a GFP-expressing control as described above.

Low viral titer: Viral plasmids are large, and occasionally rearrange during bacterial expression. Also, calcium phosphate transfection is highly sensitive to pH, and this parameter should be regularly verified. Finally, 293T cells occasionally lose their ability to amplify viral plasmids, and should not be continuously cultivated.

Unexplained cell death, slow growth, or senescence: Primary cell cultures are an excellent source of mycoplasma. New cultures should be tested prior to storage.

Unusual period length or period length variance: Since the circadian clock is temperature-overcompensated, increasing incubator temperature results in longer period lengths. (Even 0.5℃ causes a period change of 30-60 minutes.) Hence, it is important that the incubator temperature be maintained rigidly constant for all except phase entrainment experiments. Abnormally high or low concentrations of serum also alter period length, but this effect is relatively minor within standard ranges (5-20%).

DISCUSSION

The protocols used above measure circadian gene expression in adult dermal fibroblasts. In general, in both humans and mice, we have observed a correlation between the period length of this gene expression and behavioural phase (in humans) and the period of free-running locomotor activity (in mice). An important question to discuss, though, is how good this correlation is (or isn't).

At a cellular level, period length in both the SCN and peripheral tissues is stochastic: individual SCN neurons in culture and fibroblasts in culture express circadian rhythms of widely different phases and different period length (Nagoshi et al., 2004; Welsh et al., 1995). Luciferase-based measurement of fibroblasts as above yields a population-based measurement of the added luminescence of all cells in the plate. These differences are therefore not apparent in the overall average. At a population level, in most cases the mean of the circadian period length of SCN neurons or cultured fibroblasts is very similar to the mean of the period length of behavioral rhythms in the same strain of mouse (Liu et al., 1997; Yagita et al., 2001).

However, for individual cells, cell-to-cell variance is much greater in fibroblasts and dissociated SCN neurons than it is in intact SCN slices. A possible explanation of this difference is that coupling occurs in SCN tissue that is lacking in dissociated neurons or in fibroblasts in culture. In this way, aberrances in individual cells are "corrected" by input from neighbors. Mathematical modelling of simple oscillator systems suggests that such mechanisms can have major stabilizing effects. As a result, with many clock gene mutations in mice, period length in fibroblasts or in dissociated SCN neurons is often more perturbed than in either intact brain slices or in animal behaviour. For periods within "normal" ranges, however, very good correlations are usually observed (Pagani et al., submitted).

Coupling of SCN neurons occurs in multiple ways: conventional synapses, likely GABAergic (Wagner et al., 1997); electrical synapses (i.e. gap junctions) (Colwell, 2000); and neuropeptidergic coupling via vasoactive intestinal peptide (VIP) and its receptor, VPAC2 (Colwell et al., 2003; Cutler et al., 2003). Recent evidence suggests that neuropeptidergic signalling plays a dominant role, since mutations that impair it result in severely decreased precision similar to what is observed in dissociated SCN neurons, and

amplify the severity of point mutations in clock genes (Liu et al., 2007; Maywood et al., 2006).

As we have presented it, the primary utility of peripheral circadian clocks is to screen more easily for circadian differences between sets of human subjects. Hence, practically speaking, the differences mentioned above could even help: the effects of any given clock variations would be expected to be larger as observed in peripheral cells than as observed in behaviour.

Relationship between the circadian oscillator and other pathologies

A cellular method such as the one presented above, in which fundamental clock properties could be determined easily and precisely, could potentially permit an unbiased dissection of the relationship of circadian clock defects to unrelated pathologies such as depressive and affective disorders. As discussed in the introduction, these maladies are frequently accompanied by severe alterations in the timing of sleep episodes. Whether these are directly linked to changes in the circadian clock is difficult to establish by conventional means. By comparing cellular clock properties, it would be possible to find out if genetic alterations in the circadian clock are risk factors for these diseases.

Ex vivo models

Another big advantage of cellular circadian models is the possibility to manipulate the human genome. It is possible in fact to re-create the genetic mutation of a disease to investigate the molecular mechanisms that determine certain behaviour. Vanselow and colleagues introduced a mutation in *Per2,* believed to be responsible for human FASPS, into fibroblasts, and were able to recapitulate the phase advance in the behavior of FASPS patients as an advanced phase of clock-gene transcription in synchronized FASPS fibroblasts. Subsequent molecular analyses allowed them to show effects of this mutation upon phosphorylation at multiple sites in the PER2 protein, and to further demonstrate that these modifications affected both PER2 protein stability and nuclear localization (Vanselow et al., 2006).

Similarly, although fibroblasts are not themselves photosensitive, introduction of photopigments renders their circadian clocks sensitive to light. Exogenously supplied melanopsin acts as a sensory photopigment in fibroblasts and other cell types, where it

probably signals via a native G-protein signalling cascade to activate the calcium/cAMP-responsive element-binding protein (CREB) (Melyan et al., 2005; Panda et al., 2005; Peirson and Foster, 2006; Qiu et al., 2005). Transcriptionally active phospho-CREB then binds to *Per1* and *Per2* promoter CRE sites and activates transcription, thereby altering the phase of the molecular oscillator (Lee et al., 2001; Meijer and Schwartz, 2003).

Future potential

Both for the circadian clock and for other systems, it is increasingly apparent that specific molecular defects cause a wide range of alterations in human behavior and physiology. Genetic studies have linked specific diseases to a wide range of cellular signaling pathways, and many of these are – like the circadian oscillator – conserved at a cellular level. Thus, in principle, they could be accessible to the same types of cellular analyses discussed above. Given the difficulties that revolve around the collection of human tissues, *ex vivo* analyses such as these could play an important future role in determining how molecular differences at an individual level contribute to variations in many aspects of physiology and behaviour.

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6.2 APPENDIX 2

SYSTEMIC AND CELLULAR REFLECTIONS ON AGEING AND THE CIRADIAN OSCILLATOR

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ABSTRACT

From circulation to digestion to excretion, a circadian clock synchronizes most aspects of mammalian physiology with the solar day. During normal ageing, this daily coordination gradually erodes, and during pathological ageing such erosion is exacerbated. Recent experiments suggest that therapies aimed at sustaining circadian function increase quality of life in elderly patients. Hence, a better understanding of the interactions between the circadian clock and ageing – at both cellular and systemic levels – could lead to direct benefits for aged individuals.

INTRODUCTION

From archaebacteria to humans, a biological clock has governed most aspects of cellular and systemic function. In mammals, ten percent of all genes are expressed rhythmically in daily fashion, coordinated with the solar day. Hence, it is not surprising that nearly all aspects of physiology and behavior are governed by this “circadian” oscillator [1]. Within the circulatory system, heartbeat and blood pressure vary in diurnal fashion, and rhythmic expression of genes involved in fibrinolysis are believed to contribute to the prevalence of infarctus at morning hours [2]. Similarly, circadian detoxication of many xenobiotic substances is controlled by cyclic expression of cytochrome enzymes [3]; and daily variations in mood and alertness might be explained in part by circadian expression of ion channels, neuronal receptors, and hormones [4,5].

As the body ages, this coordination deteriorates in universal fashion: the circadian organization of the sleep-wake cycle is disrupted, and with it most other physiological manifestations of the circadian clock become less pronounced [6]. In situations of pathological ageing such as dementia and Huntington’s and Parkinson’s Diseases, initial disease stages are marked by abnormal daily behavior, and later stages are correlated with almost total loss of circadian function [7,8]. An understanding of this deterioration could therefore be of great assistance in increasing the quality of life for elderly individuals. Incorrectly, many of these disturbances are ascribed simply to “sleep difficulties”: in western countries one in five elderly people reports taking a sleep medication regularly [9]. While these may provide a momentary alleviation of fatigue, underlying causes remain unaddressed, leading to a chronic dependence upon these substances.

HOW IS CIRCADIAN FUNCTION REGULATED?

In all mammals including humans, the suprachiasmatic nucleus (SCN) of the brain hypothalamus acts as a central clock tissue, coordinating all aspects of overt daily physiology and behavior. However, its mechanism is cell-autonomous: each individual neuron of the SCN possesses a separate functional oscillator at the cellular level [10]. The individual clocks in each neuron are kept in phase with one another by intercellular communication involving neuropeptidergic signaling and electrical synapses [11,12]. The nucleus as a whole then synchronizes circadian timing throughout the body, probably through a combination of diffusible factors and direct connections to other brain regions

[13,14]. In fact, “slave” oscillators of similar or identical molecular mechanism to those in the SCN are present throughout the body as well. Dissociated cells of peripheral tissues such as fibroblasts appear to be able to keep time as accurately as SCN neurons in culture [15]. Regulation of daily physiology and behavior is probably a product of systemic regulation of some processes directly by the SCN, and indirect regulation of others directly by cellular clocks in other tissues [16]. Such a system has the potential benefit of temporary uncoupling between SCN and peripheral clocks. For example, if rodents are fed consistently at an abnormal time, their peripheral clocks in the liver, kidney, and other organs will change their circadian phase to reflect the new feeding time, but the SCN will continue to remain in synchrony with the solar day [17,18].

At a cellular level, the mammalian circadian oscillator is composed of interlocking feedback loops of transcription and translation. A variety of dedicated genes are used for this purpose: the products of the Clock and Bmal1 loci activate transcription of a set of afternoon-active genes including the Period and Cryptochrome family via cis-acting E-box elements present in the promoter regions of these genes. PER and CRY proteins then oligomerize to form a repressive complex that counteracts CLOCK:BMAL1 activation and shuts off their own transcription, so that the cycle can begin anew. Added robustness and control of this mechanism is provided by secondary feedback loops of transcription factors (e.g. the nuclear hormone receptors REV-ERB- and ROR-□); by phosphorylation (e.g. casein kinases 1 and 2, GSK3, and likely multiple others); and by chromatin modifying factors, RNA-binding proteins, and cellular metabolic components like cAMP. [19]. Elegantly, because this clockwork is present in most cells of the body, it is also one way that is used to control circadian output directly: the same elements critical for transcription of clock genes are also present in the promoters of many clock-controlled genes (CCGs), where they drive circadian transcription using proteins from the clock itself [20].

ENTRAINMENT

To better understand how circadian synchrony might be impaired in older individuals, it is important to understand how it is entrained in the first place. In the absence of external signals that entrain the clock, the mammalian circadian clock will “free-run” at its endogenous genetically-programmed circadian period. This period is approximately, but not exactly 24 hours. (In fact, that is why this biological clock is called “circadian” – from the Latin circa diem, or around a day.) Thus, without external timing cues, an organism will

eventually drift out of synchrony with the actual solar day. Preventing this are entrainment signals that come from the environment. In mammals, entrainment of the central clock in the SCN is a predominantly ocular process. Environmental light is transduced from the retina via both conventional rod and cone photoreceptors containing the photopigment rhodopsin, and via a special class of retinal ganglion cells containing the pigment melanopsin [21]. These cells project directly to the suprachiasmatic nucleus. Without these retinal cells – e.g. in some totally blind or enucleated individuals – the circadian clock “free-runs”, leaving affected people with chronic jetlag-like symptoms [22].

Light transduction through the lens of the eye is reduced in elderly individuals, particularly in the short-wavelength range (<480nm) [23]. Elderly mice show much smaller light-induced clock changes (“phase shifting”) than young ones [24], and elderly individuals attenuated phase advances by ocular bright light exposure in the morning [25]. In addition, there is a loss of retinal ganglion cells that transmit light signals to the circadian timing system in older animals of multiple species [26]. Hence, it has been speculated that one cause of circadian disturbances in elderly subjects is an inability to entrain properly to the environment. Such problems might be particularly acute in a clinical setting, where lighting can be irregular or even constant (e.g. the hospital ICU), or during nursing care when patients receive little natural light. Recent human studies provide evidence for this hypothesis, and show that the human circadian system is less sensitive to light in the elderly [27,28]. Moreover, practically speaking, bright diurnal lighting in nursing homes has improved behavioral circadian rhythms in residents and improved sleep quality [29], as well as cognitive performance and mood [30]. Similar results are seen for demented individuals [31]. By contrast, constant bright lighting might also have deleterious effects: at least in animal models, constant light can desynchronize SCN neurons [32], and, as discussed at the end of this review, impaired clockwork is linked to a variety of pathologies including cancer and immune dysfunction.

The degree to which brain- and eye-related entrainment changes in the elderly are mirrored or relayed in peripheral tissues remains controversial: age-dependent changes in clock gene amplitude (e.g. Per2 and Per3 genes) can be observed in blood leucocytes and oral keratinocyes, but no age-dependent differences in phase-shifting in these cells [33,34]. This question of entrainment of peripheral organs is discussed more fully next. It is

clear, though, that relatively inexpensive and easy benefits can be obtained by insuring that elderly patients under clinical care receive adequate light in circadian fashion.

SYSTEMIC CONSIDERATIONS

As mentioned already, the SCN “master clock” is entrained by light, and it in turn entrains “slave” oscillators in most cells of the body via a myriad of redundant cues. One important class is nervous signals. The neurons of the SCN demonstrate spontaneous firing patterns in daily fashion [10,35], and project to many other brain nuclei [13], where they are presumed to be important for sleep-wake cycles and cognitive function. Paradoxically, however, the first line of communication from the SCN to centers controlling locomotor activity is probably hormonal: animal experiments show that implantation of SCN neurons encapsulated in porous plastic are still capable of rescuing rhythmicity in SCN-lesioned animals [14]. In peripheral tissues, the sympathetic nervous system also plays an important role, and has been shown to communicate timing signals directly to the adrenal gland and other tissues [36]. This signaling methodology is likely one of the methods by which daily rhythms of corticosterone synthesis are generated [37].

A second class of signals is indirect products of the regulation of other brain centers by the SCN. Body temperature is one important class: even though daily body temperature varies by only 1-4 degrees C in mammals, these faint daily fluctuations – probably controlled by SCN innervation of the preoptic anterior hypothalamus and by daily activity patterns – are sufficient to entrain peripheral tissues [38]. Similarly, daily patterns of feeding are likely cues for the entrainment of peripheral clocks in tissues throughout the body [17,18], as well as a separate “food-entrainable” brain oscillator that can control locomotor activity in the absence of the known circadian clock [39,40]. In animals, alterations of either of these classes of signals effectively “decouples” the central clock from oscillators in other tissues: either reversal of daily body temperature rhythms [38] or of daily feeding rhythms [17,18] can inverse the timing of local clocks in peripheral organs. The exact mechanism of this regulation remains unknown, but recent research proposes direct molecular coupling between metabolic cycles and the circadian oscillator via redox-regulated “sirtuin” proteins [41,42], and such a metabolic link could be a plausible signal, at least for food-based entrainment. In reverse, brain-regulated corticosterone rhythms probably serve as a stabilizing influence: in animals lacking the glucocorticoid receptor in the liver, liver clocks are much more rapidly shifted by alterations in daily feeding patterns [43].

From these experiments, one can speculate that another way in which circadian oscillations might be dampened in elderly individuals might be through alterations of systemic entrainment pathways. For example, in one study aged rats showed normal entrainment of the SCN by light, but severely disrupted liver clock entrainment [44]. We hypothesize that well-characterized alterations in the hypothalamic-adrenal-pituitary (HPA) axis in elderly individuals might play a major role. For example, circadian oscillation of cortisol is dampened, peak levels are reduced, and evening levels are increased in older people [45]. This condition would act synergistically: not only would the direct circadian effects of cortisol upon digestion and detoxification be lost, but also any effects of irregular feeding and body temperature would have even greater effects upon the circadian oscillator because of impairment of parallel cortisol-based entrainment mechanisms. Multiple studies have shown that imposed regular routines of mealtime and exercise improve circadian consolidation of sleep-wake cycles in elderly individuals [29,46]. Physiologically speaking, meal and light routines might help substitute for the loss of internal circadian signals, and themselves act as timing cues. Under normal conditions in young, healthy individuals, factors such as meal timing are overpowered by systemic circadian cues, and therefore might play a less important role. (A similar situation exists in blind individuals: in the absence of the strong influence of ocular light, many subjects can be entrained to the 24-hour day by weak nonphotic timing cues that do not play a significant role for sighted individuals. [47]).

CELLULAR CONSIDERATIONS

As already described, virtually all cells in mammals have an independent circadian clock capable of sustained oscillations in isolation. Because each cellular oscillator is slightly different, though, circadian oscillations in dispersed cultures or tissue slices of peripheral cells rapidly dampen. The function of the redundant signaling described above is to synchronize all cellular clocks, thereby sustaining robust rhythms of gene expression and physiology at the tissue level. Recent studies suggest that there is a second function, though: to drive a subset of cellular circadian gene expression directly. Thus, genetically modified mice lacking a functional circadian clock in a particular organ lose circadian expression of a subset of genes (those coupled to the cellular oscillator) but still show circadian expression of others (those coupled to systemic mechanisms) [48,49]. Similarly,

tissue-specific rescue of a clock gene results in rescue of only a subset of physiological aspects of the underlying mutant strain [50].

The cellular heterogeneity of clock properties probably serves a definite function. Although the day length on our planet is fixed to 24 hours, the period of light during this time can vary dramatically in lateral clines. To cope with these differences, the circadian clock is able to track both light onset (dawn) and light offset (dusk). Because of cellular differences in the SCN, some of its cellular clocks are phased earlier, and thereby “track” dawn, and others are phased later to track dusk – a hypothesis formed long before the existence of cellular clocks was discovered [51], and recently confirmed in rodent SCN neural activity studies [52] and luciferase imaging experiments [53]. To keep SCN cells locally synchronized, neurochemical coupling mechanisms are used [12,54], and probably gap junctions as well [55]. Elimination of these components in mice results in dramatically dampened circadian oscillations.

These cellular mechanisms comprise yet another way in which circadian rhythms could be altered in older individuals. It is possible that cellular clocks are themselves altered in aged cells, either intrinsically or due to increased release of inflammatory cytokines which are known to affect circadian gene expression [56,57]. Alternatively, changes in synaptic patterns and neurochemical coupling within the SCN could also result in alterations in cellular clock function [58,59]. Lending credence to this hypothesis, SCN electrical firing shows reduced circadian amplitude in older rats [60,61], and reduced sensitivity to the hormone melatonin [62]. Transplantation of a fetal SCN into aged hamsters improved behavioral rhythmicity [63,64].

CIRCADIAN ALTERATIONS IN THE ELDERLY: WORKING BACKWARDS

A great deal of research has been done to characterize the specific physiological changes that occur as humans age, and some of these have already been mentioned above. The nature of these changes could permit us to understand better which of the mechanisms presented above might be responsible for the decline in circadian function in elderly individuals.

Even based upon simple questionnaires, it is clear that from adolescence onward, human circadian clocks move earlier as individuals age. Between the ages of 20 and 80 years, the

timing of sleep shifts an average of two hours [65]. At the same time, sleep consolidation – i.e. the ability to sleep in long, unbroken nighttime bouts – is decreased. In addition, the total amount of slow-wave “deep” sleep decreases, and rapid-eye-movement “dream” sleep is more evenly distributed [66]. In short, there is a marked decrease in the “circadian amplitude” of sleep. Sleep patterns are believed to be the sum of two independent processes: a circadian one that programs human beings to sleep preferentially at night, and a homeostatic one that increases sleep pressure with increasing time awake [67]. In principle, the fragmentation of sleep could be caused either by a reduction in the strength of the circadian component, or by a change in the homeostatic component, and this question is actively debated at present.

The reduction in circadian amplitude is not limited to sleep: it is universal, and results in a variety of endocrine consequences. Appetite is reduced during meals and inter-meal eating increases, and activity is increased during nighttime hours, especially during pathological ageing: dementia, Huntington’s, and Parkinson’s disease. Meanwhile, the circadian amplitude and magnitude of many hormones are reduced, especially melatonin, prolactin, and those of the HPA axis, as well as glucose [68]. Although some studies are contradictory on this point, body temperature may be both lower and oscillate with lower circadian amplitude [69]. Endothelial nitric oxide oscillations are also reduced, and may be responsible for the damping of circadian cardiac function [70]. In short, it is easy to imagine how circadian physiology might be attenuated.

A thornier problem is what might shift phase. One obvious hypothesis to explain earlier phase would be a shortening of the period of the circadian oscillator. Intrinsic period shortening has been observed in aged rodents and nonhuman primates by some groups [71,72]. In humans, laboratory studies make this hypothesis unlikely: cross-sectional studies of younger and older adults under “forced desynchrony” – artificial lighting conditions that measure the intrinsic period of the circadian oscillator unaffected by external environment -- show no differences in average period length [73]. Interestingly, the same studies also show that the timing (“phase angle”) of sleep relative to the circadian clock (measured via the pattern of secretion of the circadian hormone melatonin) is earlier, and sleep is less consolidated [74,75]. It is this lack of sleep consolidation that has led other groups to speculate that sleep fragmentation is itself responsible for the circadian phaseshift [76].

According to this idea, age-related changes in sleep structure and sleep consolidation reflect a reduction in the circadian force that opposes homeostatic sleep pressure [77]. This influence is particularly significant in the late evening, when the circadian drive for wakefulness is highest (i.e., the wake maintenance zone), and in the early morning hours when the circadian drive for sleep is at its maximum, thus leading to more sleep episodes in the evening and earlier morning wakening in elderly individuals. As consequence of this altered sleep-wake pattern, the elderly may expose themselves to more morning than evening light, which may result in an earlier circadian phase.

This model works well in normal conditions, but it does not fully explain similar results studying sleep latency under “forced desynchrony” conditions that look at the free-running circadian oscillator [73]. We suggest that this paradox might be explained by hormonal changes, and that the same hormonal differences might also explain the reduced “circadian drive” discussed previously. Since many hormones phase-shift peripheral circadian clocks, alterations in their overall levels would be predicted to alter period in “free-running” conditions, and phase in an entrained environment. However, if these hormones do not act upon the SCN – due to the blood-brain barrier or a lack of the appropriate receptors – the result would be a shift in the phase of circadian physiology (controlled most directly by peripheral organs and brain regions) without a change in the free-running period of circadian behavior (controlled by the SCN). (See Figure 1.)

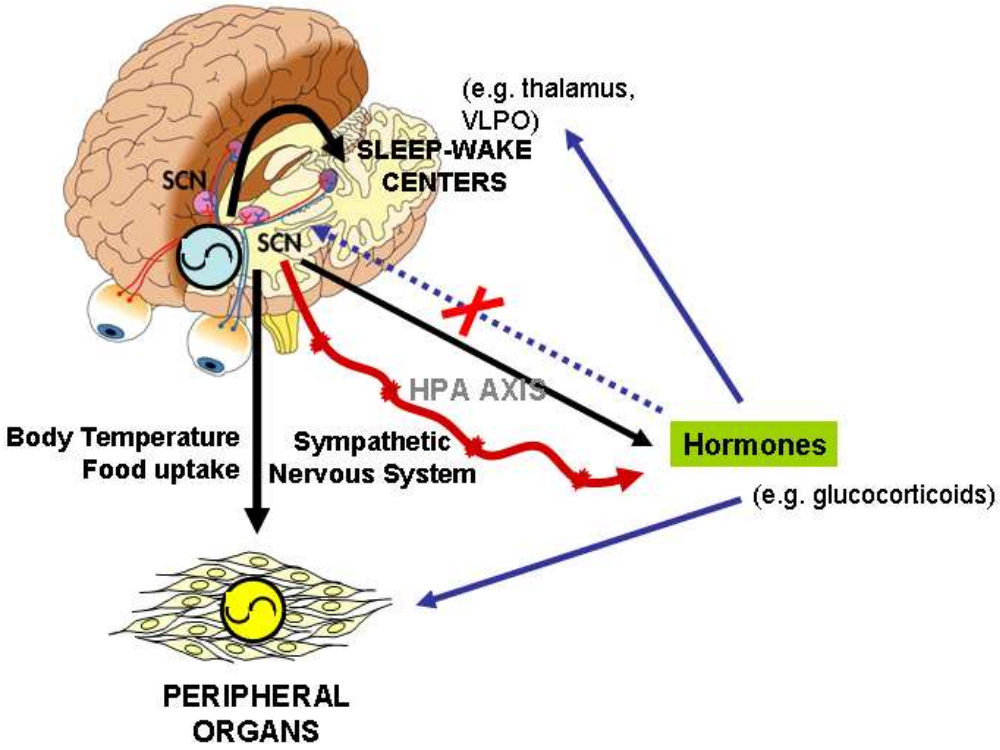

FIGURE 1. Interaction between master and peripheral clocks. In mammals, the master clock in the suprachiasmatic nucleus communicates timing information to other tissues in the brain and body via a redundant combination of signals: nervous innervation of other brain regions, secreted hormones, and indirect cues like body temperature and food intake (shown as black arrows leading out from the SCN), as well as by sympathetic innervation of peripheral organs (red arrow). Hormonal signals that influence circadian phase can in turn affect both peripheral organs and sleep-wake centers (blue arrows). We suggest that some of these hormones (e.g. glucocorticoids) cannot signal to the SCN (dashed blue arrow), though they can still affect sleep-wake centers. In older individuals, the circadian amplitude of signals from the SCN is decreased, resulting in sleep fragmentation and reduced amplitudes of circadian behavior and physiology. We propose that at the same time, changes in hormonal balance -- perhaps in the hypothalamic-pituitary-adrenal axis -- would additionally alter the circadian phase of behavior. Because these hormones do not "feed back" to the SCN, they would not change circadian period length under free-running conditions.

PATHOLOGICAL EFFECTS OF REDUCED CIRCADIAN FUNCTION IN OLDER INDIVIDUALS

The obvious effect of decreased circadian amplitude is a decreased "quality of life", probably driven by worse sleep and a feeling of less energy during active periods. An increasing number of mouse studies suggest that decreased circadian function might also have a direct effect upon lifespan. These studies fall into three classes. First, there are mouse studies showing that deletions of some clock genes (e.g. Bmal1, Per2) lead to decreased lifespan and increased cancer and other age-related pathologies [78,79]. Though interesting, the metabolic defects linked to deletions of these genes makes it unclear if their effects are gene-specific or clock-specific. Secondly, there are studies suggesting that circadian desynchrony per se – via chronic jetlag paradigms, for example

– lead to decreased lifespan [80] and increased cancer rate and tumor growth [81]. Finally, an increasing number of mechanistic studies tie the circadian clock intimately to questions of metabolism. They show for example that the circadian clock times cell division in adult animals [82], and that it regulates and is regulated by proteins sensitive to cellular reduction and oxidation like sirtuins, which have themselves garnered a great deal of interest as anti-ageing proteins [83]. Thus, the circadian clock may be fundamentally tied to the balance of factors that prevent cellular damage, and reduced circadian amplitude may therefore accelerate this process.

Although not discussed herein, other pathological effects may also arise separately through the changes in sleep that come about during ageing. For example, sleep deprivation results in immune system dysfunction and chronic inflammation. Since these two effects are commonly observed in aged individuals who suffer from disturbed sleep, it is easy to hypothesize a causative link [84]. Other studies, however, suggest that according to cognitive parameters, healthy subjects actually tolerate sleep deprivation better than younger ones, belying the idea that they are “sleep-deprived” because of their altered sleep structure [85]. Further research is clearly needed upon this interesting question.

PERSPECTIVE ON EXPERIMENTS AND TREATMENTS

Because the synchronization of peripheral circadian oscillators can be driven by indirect cues such as mealtime and body temperature, some of the best current circadian therapies for elderly individuals may also be the simplest: timed regular activities and mealtimes, and bright daytime light. Multiple reports from clinical settings already suggest that these measures can help. More long-term, the susceptibility of peripheral clocks to multiple hormones may make hormonal therapies another viable option. Exactly which hormones might be effective – and which, if any, might be responsible for these changes in the first place – are experimentally testable hypotheses, particularly given easy access to peripheral clocks in some human tissues. Thus, it is likely that future experiments can not only help illuminate the causes of circadian dysfunction in the elderly, but also help alleviate it in a safe manner.

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