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**REVIEW**

# Regulation of circadian rhythms

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**Summary**
The human circadian system is evidently regulated by components which can be found in the retina (light input), a suprachiasmatic nucleus in the hypothalamus (clock genes) and the pineal gland (melatonin synthesis). Clock genes are interdependent through two intracellular feedback loops. The pineal gland is not the single important producer of melatonin, as immune cells can also produce this hormone. Immune cells contain active clock genes as SCN cells and we can suggest that the regulation of the circadian system is a component of the neuroimmune regulation of the organism. The endogenous character is dominant in SCN, which is modulated by darkness and which synchronizes organisms to the light/dark regime including immunity. The exogenous character seems to be dominant in the immune system which synchronizes the organism including SCN cells to other environmental stimuli. The mathematical theory of chaos shows that the circadian activity of a cell is derived from ultradian metabolic rhythms; these rhythms support the stability of living systems which can be changed by a limited repertoire of interventions. The complexity of neuroimmune interactions perhaps explains why we are far from knowing the mechanism concerning the regulation of biorhythms despite the vast number of related scientific publications.

*Keywords:* circadian rhythm – chaos – clock gene – suprachiasmatic nucleus – melatonin – immune system

## INTRODUCTION

Life is a hierarchical structure of periodic processes (Fig. 1) including metabolism, replication and self-organization. As temporal characteristics of the environment are structured by cyclic changes, i.e. resources and enemies change periodically, endogenous biological clocks have developed during evolution. The endogenous nature of biorhythms was first observed by Jean Jacques d'Ortous de Mairan and presented in a lecture by Marchant in 1729 (Berger 1980). Biorhythms are periodic changes in living organisms which can be described by their period, amplitude, mesor, and acrophase (Haus and Touitou 1994).

Biorhythms have a wide spectrum of period times: from fractions of 1Hz (circasemicentennial) to 10-year (circadecennial) cycles (see Halberg et al. 2000, for review). Rhythms with a period of approx. 24 hrs are called circadian (circa=about, dian=day; 'diurnal' is synonymous with 'circadian'. Ultradian rhythms have a period shorter than 20 hrs (and higher frequencies than circadian), they are a consequence of metabolic oscillations and we can assume that they are not directly induced or regulated by external stimuli related to periodic

changes in the environment. Infradian biorhythms have a period longer than 28 hrs; the period of circannual rhythms is approx. 1 year. The most studied biorhythms are circadian oscillations. They are an evolutionary adaptation for periodic changes in daylight, temperature and many other environmental factors as a consequence of the Earth's rotation (cf. Sharma 2003); these oscillations are important for human health, they can be keys to the synchronization of other biorhythms.

Angular frequency is the frequency of a periodic process usually expressed in degrees per unit of time; the angular frequency of circadian rhythms is 15°/hr.

Mesor is the rhythm adjusted mean. Amplitude is the extent of an oscillation, it measures one half of the extent of rhythmic change in a cycle, e.g. the difference between maximum and mesor of a best fitting cosine curve. High amplitude in human subjects has usually high clinical significance and the data of these characteristics must be related to controls measured at the same time. A small amplitude can be theoretically interesting but clinically unimportant; clinically insignificant is, for example, the circadian rhythm of erythrocyte counts (Haus 1996, Berger 1987). Both the amplitude and mesor of many circadian rhythms decrease in the elderly (reviewed by Berger 2003).

Acrophase is the measure of timing, it is the highest point of the fitted periodic curve (units: time units, angular measure), it can be different from a measured peak. Similar positions of the acrophase are usually found in species with prevailing running activity during the day (= diurnal animals) that is during a light period in a vivarium (Haus 1996, Plytycz and Seljelid 1997) while the course of the circadian rhythm in nocturnal species is the opposite (e.g. Denison and Zarrow 1954, Berger 1983, Perpoint et al. 1995).

Many papers on regulation of circadian rhythms have been published: for example using both the terms 'regulation' and 'circadian', a search of the database at ISI Web of Knowledge indexed almost 2.5 thousand articles published during the last twenty years and almost 1.3 thousand papers during only the last five years. The aim of this study is to explain why we do not know the mechanism of circadian rhythm regulation, although a vast number of studies on this topic has been published.

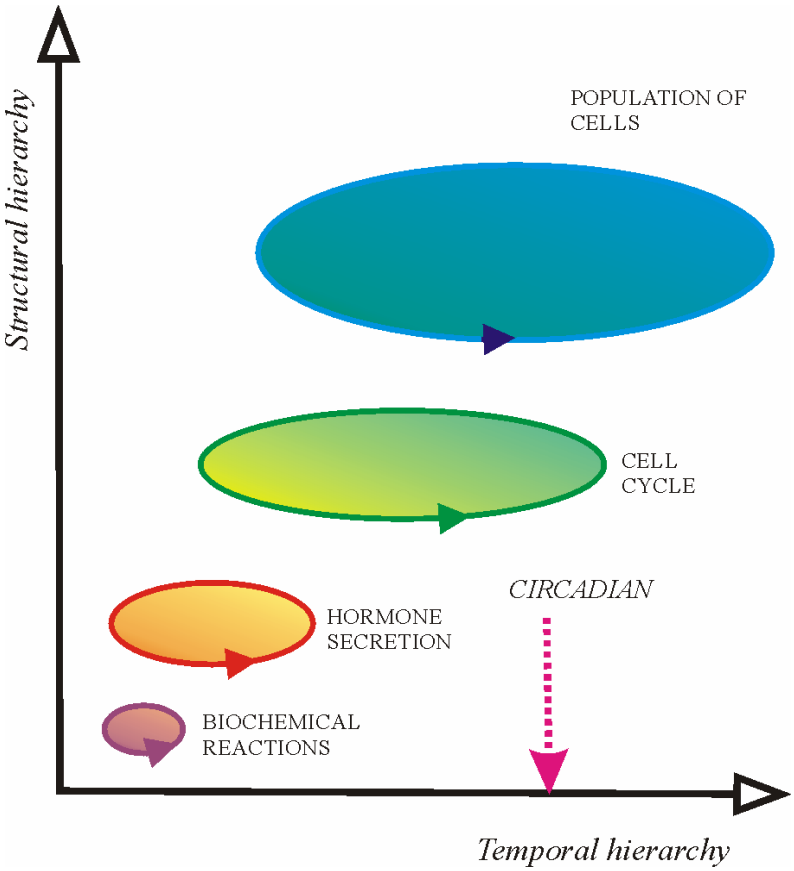


Fig. 1. **Hierarchy in living systems** (Lloyd and Edwards 1987, modified)

## CELL CLOCK

Circadian rhythms are obvious in both unicellular and multicellular organisms, in both plants and animals, and they are very important for human physiology, too. Common elements of the circadian clock, cover genes transcribed in feedback loops. Clock gene proteins influence the transcription of clock controlled genes.

The relationship between genetic information and the regulation of circadian rhythms was documented during the sixties by the discovery of mutants which have various stable periods different from circadian oscillations in wild organisms; the first clock mutants were found in Neurospora (Neurath and Berliner 1964). The hypothesis on gene regulation of circadian rhythms, inspired by Jacob's and Monod's operone model, was firstly postulated by Ehret and Trucco (1967); they suggested the existence of the so-called "chronon", the set of linearly transcribed cistrons which regulate one period of the rhythm.

The importance of the discovery of gen per (from 'period') in *Drosophila* by Konopka and Benzer (1971) was not recognised immediately and several other models (see Berger 1980 for review) were also suggested at that time. Currently, the important role of the so-called clock genes for the regulation of biorhythms is well accepted; we know that rhythms are the result of clock gene expression and synchronization by environmental and endogenous influences, mostly by light.

Information on daylight or its imitation by artificial illumination is received by retina cells and project via the retinohypothalamic tract into the suprachiasmatic nucleus (SCN) in the hypothalamus. Special light sensitive ganglion cells act in the retina as brightness receptors and send appropriate information useful for the regulation of circadian rhythms (Forster 2004). A circadian clock seems to consist of two interdependent transcriptions at feedback loops: the first loop controls *per* expression, the second feedback loop *(d)clk* expression; clk RNA oscillations are roughly antiphase to that of *per*. The second feedback loop is not necessary for molecular or behavioural rhythms; it can be necessary for (i) robust high-amplitude rhythms required for survival in the natural environment (but dispensable in constant conditions), (ii) transduction of various environmental signals to the first feedback loop, or (iii) the control of the expression of time-specific circadian outputs (Allada 2003).

The molecular circadian clock is evolutionarily highly conserved, i.e. it is very similar for many species from insects to mammals. *Drosophila* is the most frequently studied model. Circadian behavioural rhythms in *Drosophila* are controlled by genes *per* and *tim* (timeless). Around dawn, proteins PER and TIM down regulate them by suppressing the activation of transcription factors dCLK (dCLOCK) and CYC (CYCLE, BMAL1 in mammals) which form a heterodimeric complex. VRI (VRILLE, a basic leucine zipper transcription factor) accumulates and represses *clk.* PDP1ε accumulates and activates *clk*. At dusk, dCLC/CYC activates *per, tim, vri*, and *pdp1* genes, PER levels increases. VRI accumulates earlier (peaking around dusk) and disappears earlier than PDP1ε (Cyran et al. 2003). The expression of *vri* and *pdp1*ε (PAR domain protein, PAR=proline and acidic rich), like *per* and *tim*, is dependent on *clk* (Cyran et al. 2003). At dusk, CLCK/CYC activates *per, tim, vri*, and *pdp1*ε. The cytoplasmic interaction between PER and TIM, which precede their nuclear translocation, is delayed by a kinases DBT and CKII. DBT and CKII phosphorylate PER, which lead to PER repression activity (Nawathean and Rosbash 2004). The interaction between PER and TIM follows a several-hour accumulation of TIM and the complex PER/TIM blocks DBT (Young 1999). Synchronization of rhythmic gene expression to the sunlight cycle involves *cry* expression.

In mammals (Fig. 2), the first loop comprises *per*, the second is based on the *cyc* homolog *bmal1*. Darkness starts *per* gene expression in SCN cells. PER1 and PER2 proteins are phosphorylated in the cytoplasm by CKIε protein, then PER and CRY (CRYPTOCHROME, blue light photoreceptor) form heterodimer that is translocated into nucleus and inhibits transcription of their own genes, while hetorodimer CLOCK/BMAL1 represents the transcription activator of the *per* and *cry* promoter. The transcription of mammalian *per1* and *cry2* clock genes increases during the night, the expression of *per3* and *cry1* clock genes damps and abolishes in constant darkness (Simonneaux et al. 2004). CKIε may affect nuclear translocation and life of mPER. REV-ERBα plays an analogous role to VRI repressing *bmal1* transcription by binding ROREs within the *bmal1* promoter; ROREs are binding sites for REV-ERB and ROR orphan nuclear receptors (Allada 2003).

There are only minor variations: the peak of mRNA occurs at daytime, and CRY, BMAL1and PER/CRY proteins oscillate in mammals, while different parts of the molecular clocks oscillate in insects (see Badiu 2003, Kennaway 2004, Simonneaux et al. 2004 for reviews).

The internal clock in SCN seems to be dominant for mammals including men, but clock genes were also found in peripheral clocks such as liver, muscle, kidney, heart (Badiu 2003, Okamura 2003), mononuclear circulating leucocyte (Oishi et al. 1998), and bone marrow cells (Chen et al. 2000). Daily restricted feeding can produce a circadian

rhythm which is associated with the periodical expression of mouse clock genes in the cerebral cortex and hippocampus (Wakanatsu et al 2001). The relationship between peripheral clock genes and suprachiasmatic clock genes is poorly understand.

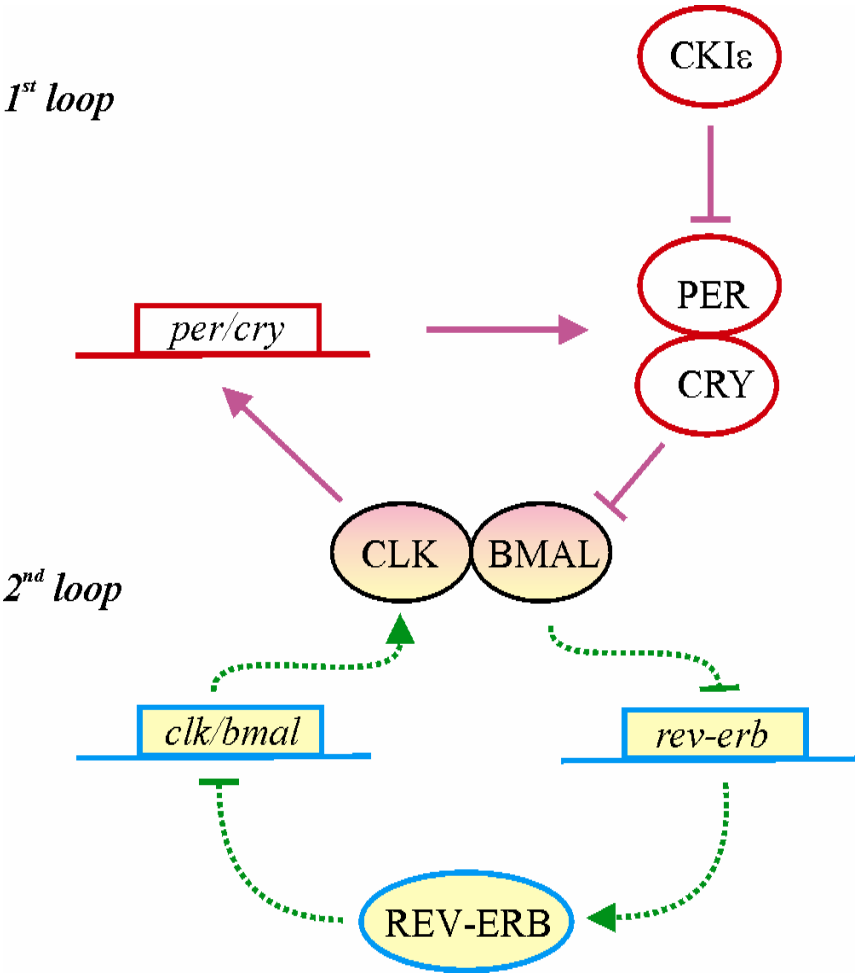


Fig. 2. **Proposed model for interdependent feedback loops in mammals circadian loops** (Allada 2003, modified)

**CHAOS**

Chaos is a mechanism which can generate random-looking time series from simple deterministic systems (Lloyd and Lloyd 1995). Oscillations in living cells express chaotic dynamics where one trajectory represents the most stable systém. A controlled chaotic attractor provides multifrequency outputs that determine rhythmic behaviour on different time scales with the facility for rapid state changes from one periodicity to another (Lloyd 1997). A change of the rhythm trajectory is possible only during a short period (Fig. 3); for example, a light pulse can change a phase of circadian rhythm only in a short portion of night. Models based on the negative feedback regulation between clock genes expression and the level of their proteins indicate that the periodic forcing of circadian oscillations by light-dark cycles can result either in entrainment to the external periodicity or in aperiodic oscillations, depending on the magnitude of the periodic changes in the light-controlled parameter (Gonze et al. 2000).

Principles of chaos control have been used with success to regulate both in vitro excitable tissues and human cardiac electrophysiological dynamics

(Glass 2001, Christini et al. 2001). Chaotic dynamics occur during the organization of intracellular macromolecular complexes (Aon et al. 2000). It seems that the products of circadian clock genes, PER and TIM proteins, are capable of generating autonomous chaotic oscillation (Leloup and Goldbeter 1999). The stochastic model based on cooperative repression of the per gene by the PER protein indicates that circadian oscillations can emerge at the cellular level, even when the maximum numbers of mRNA and protein molecules involved in the oscillations are of the order of only a few tens or hundreds (Gonze et al. 2004).

The mathematical theory of chaos suggests (Klevecz et al. 1991) that small units oscillate in the ultradian domain and when they are joined into aggregates they produce the circadian rhythm. Although Klevecz et al (1991) considered units as cells, we can suggest that these units are cellular components and they aggregate the so-called cells and present the circadian rhythms that can be observed in unicellular organisms or isolated cells in vitro. Controlled chaos (Glass et al. 1988, Steeb et al. 1995) offers stable systems which can be regulated with minimal effort in the suitable time point of its trajectory; it makes the adaptation easily with minimal energy claims. From this point of view, controlled chaos in biorhythms offers living systems more stability and adaptability. Biorhythms are, therefore, an ancient phenomena which has been found in all recent organisms. Several million years of evolution could lead to a wide diversity of control mechanisms of biorhythms including circadian rhythms; this seems to be one of the most important adaptations to the Earth's environment.

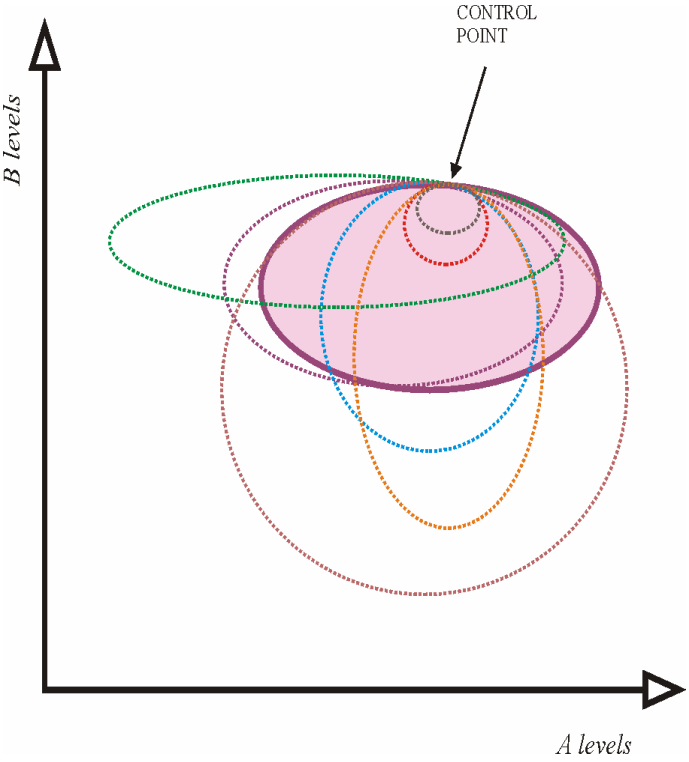


Fig. 3. **Chaos generates random-looking time series**. A change of the rhythm trajectory is possible only during a short period

## ROLE OF SCN

SCN is very important for all mammals, including man, in the regulation of circadian rhythms (Fig. 4). It is composed of multiple single-cell oscillators, which generate circadian outputs that regulate overt rhythms (Haus and Touitou 1994, Hastings et al. 2003). The circadian clock system in SCN receives stimuli generated by the light directly via the retinohypothalamic tract, some stimuli can be received following their modification in other neurons (Morin et al. 2003). It seems that ageing of

biorhythms in humans and other mammals is caused by the ability of SCN to drive oscillations in other tissues and social synchronization extending photic stimulus enhances the quality of life (Berger 2003).

In SCN, signals generated by light regulate both nuclear and cytoplasmic mitogen activated protein kinases (MAPK) (Butcher et al. 2003). MAPK are maximally phosphorylated during the day and light pulses during the night can induce rapid phosphorylation of these enzymes (Pizzio et al. 2003). MAPK phosphorylates the transcription factor BMAL1, a positive regulator for the autoregulatory feedback loop in the circadian oscillator (Sanada et al. 2002).

The location of light-responsive cells, defined by direct retinohypothalamic input and light-induced gene expression, largely overlaps with the location of non-rhythmic cells in the SCN central "core" compartment (Lee et al. 2003). Cells of the core region express lightinduced genes, the "shell" compartment of SCN contains cells with rhythmic expression of clock genes, and intra-SCN connections project from the core to the shell compartment (Kriegsfeld et al. 2004).

SCN cells release humoral factors that modulate the activity of other cells, for example cells of the pineal gland (Gillette and Mitchell 2002) and the immune system (Roberts 2000). The combination of inhibitory and stimulatory SCN outputs via the multisynaptic control of melatonin pineal synthesis could be part of the mechanism of day-length adaptations incl. seasonal changes (Perreau-Lenz et al. 2003). In contrast, humoral factors also modulate clock gene expression in SCN (Lunkkvist et al. 2002, Cardinali and Esquifino 2003), interferon-α disrupts photic induction of *per* gene in SCN (Ohdo et al. 2001).

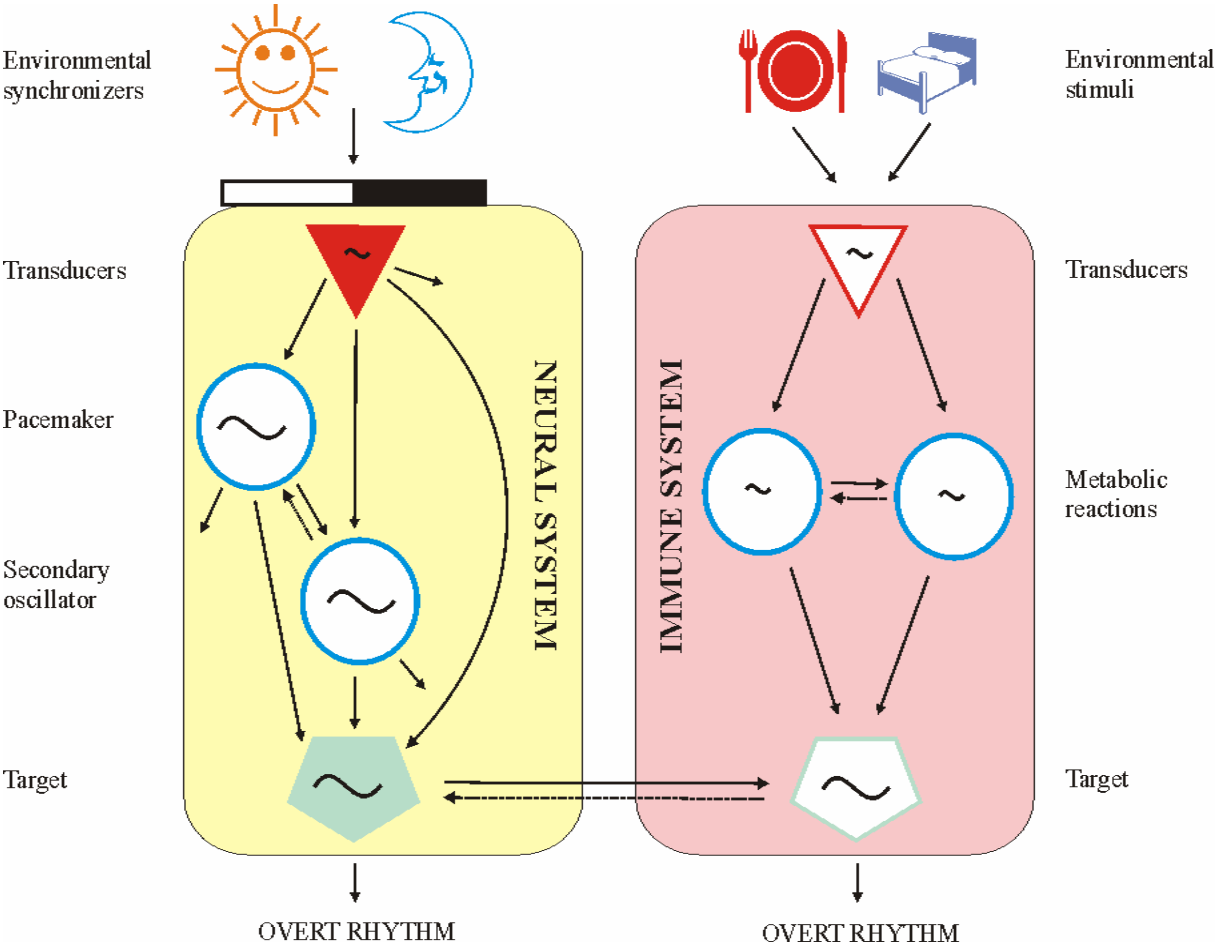


Fig 4. **Circadian timing mechanism** as the result of interactions between neural and immune system in mammals

## MELATONIN

The pineal gland synthesizes hormone melatonin (N-acetyl-5-methoxytryptamine) in at high levels in the night and it is, therefore, dubbed the hormone of darkness. Biorhythms of these hormone levels play an integrative role in synchronizing various oscillations, especially in endocrine organs. An alteration of the melatonin secretory pattern has been found in several psychiatric disorders (Pacchierotti et al 2001). Melatonin acts as an antioxidant (may be anti-ageing) and an immunomodulator; it has antitumour activity. It was found in various cells, from bacteria to humanones. Bone marrow cells (Conti et al. 2000) and circulating lymphocytes (Carrilo-Vico et al. 2004) also produce melatonin which can modulate both circadian and immune systems. Moreover, pineal gland melatonin production can be modified by many immunological factors such as antigenic stimulation, inflammation, cytokines, histamine, prostaglandins, and opioids (Skwarlo-Sonta et al. 2003)

In contrast to melatonin production in the bird pineal gland, which also oscillate in vitro, the mammalian pineal glands produce circadian oscillations of melatonin levels only if the innervation from cervical ganglion is intact (Csernus and Mess 2003).

Melatonin modulates the functions of cells which have melatonin receptors (Dubocovich et al. 2003) and, on the contrary, melatonin production is modulated by various humoral products of other cells, in particular immune cells (Finocchiaro et al. 1988, Skwarlo-Sonta 2002). Melatonin receptors belong to the G-protein coupled receptor family (receptors mt1 and mt2) and the quinone reductase family (receptor mt3) (Witt-Enderby et al. 2003). Melatonin receptors were found in both SCN cells and cells of many other organs. This hormone lowers the threshold for adenosine to induce cAMP-sensitive genes (Stehle et al. 2003).

The disruption of circadian rhythms can be associated with accelerated growth of tumours in both animals (Filipski et al. 2002) and human subjects (Fu and Lee 2003). As cycle cell kinetics is under circadian control (Merrow and Roenneberg 2004), the circadian rhythms go on the tumour suppressor. Thus, the antioncotic effect and synchronization of biorhythms following melatonin administration can be a consequence of the stabilization of the circadian system. Melatonin acts as a differentiating agent in some cancer cells, lowers their invasive character after changing their adhesion molecules, induces programmed cell death or acts as a cytostatic drug by the modulation of estrogen receptor expression, protein kinase C activity, calcium/calmodulin activity, intracellular redox status, and changes the cytoskeletal structure (cf. Blask et al. 2002); these effects cohere frequently with the suppression of tumour linoleic acid uptake and its conversion to 13-hydroxyoctadecadienoic acid which activates EGFR/MAPK mitogenic signalling. It has been shown that the expression of several genes involved in cell cycle regulation, and tumour suppression are also controlled directly by circadian regulators (Fu et al. 2002).

## CONCLUSION

The human circadian system is regulated by environmental stimuli and endogenous clocks. Special clock genes were found, and they are interdependent through two feedback loops. Central clocks are in the SCN of the hypothalamus, which regulates the production of melatonin in the pineal gland. This hormone is also produced by immune cells. Melatonin regulates SCN vice versa. The theory of chaos has documented that the circadian rhythm of cell behaviour can emerge from ultradian metabolic rhythms and this system is very stable and well regulated. It seems that biorhythms are necessary for all living systems and they are, therefore, evolutionally conservative. Mechanisms for their control are so complex that knowledge concerning them depends on the discovery of most characteristics of life; this may be the reason why we are still far from knowing them fully.

## ACKNOWLEDGEMENT

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