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# Vnitřní komunikace v rámci cirkadiánního systému a její vliv na naše zdraví

# Internal Communication within the Circadian System and its Significance for our Health

Bakalářská práce

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V Praze, 12.05.2015

Podpis

## Abstract

Mammalian circadian cycle is generated by hierarchically organized system of internal rhythmical oscillations in clock gene expression (*Clock*, *Bmal1*, *Per*, *Cry*, *Rev-Erb*, etc.) which take place in nearly all living cells in our body. The master pacemaker is located in suprachiasmatic nucleus (SCN) in hypothalamus. According to its synchronization to photic and non-photic external stimuli SCN generates signal for entrainment of peripheral clock. Peripheral clock synchronization is maintained via neuronal or hormonal (glucocorticoids, melatonin) pathways, regulation of body temperature or food intake and affects various physiological processes. Desynchronization of central and peripheral clock can be the cause or the manifestation of impaired health condition.

**Key words:** circadian rhythm, entrainment, SCN, clock genes, peripheral clock, glucocorticoids, health, neurodegenerative diseases

## Abstrakt

Savčí cirkadiánní cyklus je generovaný hierarchicky organizovaným systémem vnitřních rytmických oscilací v expresi hodinových genů (*Clock*, *Bmal1*, *Per*, *Cry*, *Rev-Erb*, etc.), které se nachází v téměř každé živé buňce našeho těla. Hlavní *pacemaker* se nachází v suprachiasmatickém jádře (SCN) v hypothalamu. V závislosti na jeho synchronizaci s vnějšími světelnými a nesvětelnými stimuly SCN generuje signál pro synchronizaci periferních hodin. Synchronizace periferních hodin je zprostředkována nervovou nebo hormonální (glukokortikoidy, melatonin) dráhou, regulací tělesné teploty nebo příjmu potravy a ovlivňuje mnoho fyziologických procesů. Desynchronizace centrálních a periferních hodin může být příčinou či projevem zhoršených zdravotních podmínek.

**Klíčová slova:** cirkadiánní rytmus, synchronizace, SCN, hodinové geny, periferní hodiny, glukokortikoidy, zdraví, neurodegenerativní onemocnění

# Obsah

# 1. Introduction

Circadian rhythm is a trait which can be observed in most living organisms around the world, from single-cell prokaryotes [1] to mammals. In mammals it could be best observed at a level of behaviour which changes according to alternation of day and night, caused by Earth's rotation around its axis while orbiting around the sun (which is cause of changing seasons). One Earth's spin takes 24 hours and organisms are equipped with endogenous timekeeping mechanism driving rhythms with a period close to the solar day. These rhythms are called "circadian" from "*circa diem*" meaning "about a day". The length of the circadian period ranges from 20 to 26 hours and may slightly vary among individuals.

Although the behavioural rhythms might seem to be a simple reflection of changes in the environment, these rhythms are endogenously controlled and thus maintained even in non-rhythmic conditions. They are driven by an autonomous mechanism generating molecular oscillations that can be found in nearly every living cell of our body [27]. The mechanism, is based on a principle which is common to all eukaryotic organisms has developed early in phylogenesis [32].

In mammals, the circadian rhythms are driven by a hierarchically organized system. Each organ in the mammalian body harbours its own peripheral circadian clock and they are all together orchestrated by the superior central clock, the master pacemaker that generates signals which synchronize these clocks in the periphery [41].

The presence of the circadian clock in the bodily cells implies, that circadian rhythmicity is expressed not only as the behavioural and sleep-wake cycles, but also at the level of many physiological processes inherent to these peripheral organs, such as heartbeat, blood pressure, renal functions or the process of digestion [41].

Recent studies have suggested that proper communication between the central and peripheral clock is one of the most important pre-requisites for maintaining good health. The desynchronization among the individual parts of the circadian system affects not only quality of life, but in some cases can be even life threatening. The aim of this work is to describe the molecular mechanism underlying generation of circadian rhythm and various ways of communication between the circadian system components and finally to clarify the importance of circadian rhythm for the state of health.

## 2. Molecular oscillations

Due to its ability to be transmitted to next generations, it was recognized, that circadian clock and its behavioural outcome has its base on genetic level. Its principle is based on changes in gene expression according to time of day. Those changes, oscillations, are the essence of circadian rhythm.

Circadian molecular oscillations in all organisms are based on a similar principle, although various systems in different organisms are maintained by different genes and proteins [32]. The genes which are essential for the clock mechanism are called clock genes. They play a role of positive factors, which promote transcription, and negative factors, which inhibit it. These factors affect each other's transcription and create the so-called feedback loop which is the basic essence of circadian oscillations [29,32]. This chapter will be mainly focused on mammalian circadian system.

The mechanism of the mammalian clock is primarily based on two feedback loops. The key participants of these feedback loops are CLOCK and BMAL1 transcriptional factors. CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT–Like Protein) are proteins belonging to bHLH-PAS (basic helix-loop-helix/Per-Arnt-Sim) family. Both are transcribed and translated in cytoplasm. These factors then aggregate and create heterodimers and are translocated back to the nucleus. After the translocation they act as positive transcriptional factors activating transcription by binding to E-box (Enhancer box) sequence found in promoter areas. They preferably bind E-boxes containing CACGTG nucleotide sequence [30]. In addition to this, CLOCK protein has intrinsic acetyltransferase activity and modulates targeted histones to promote transcription [31]. After binding to E-boxes, CLOCK/BMAL1 heterodimers activate transcription in their target genes *Period* (homologues *Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1*, *Cry2*).

Once translated, one of PER homologues and one of CRY homologues bind together forming PER/CRY heterodimer, which upon phosphorylation by CKδ/ε (casein kinase δ or ε) is transported back to nucleus [31]. Over time, the level of PER/CRY heterodimers in the nucleus increases until it reaches its threshold. Past this threshold, PER/CRY complexes begin to complete negative feedback loop as they bind CLOCK/BMAL1 complexes and thus inhibit their transcriptional activity on their own genes. Consequently, the amount of PER/CRY heterodimers is gradually decreasing. When certain level is reached, the complexes no longer inhibit transcriptional factors and CLOCK/BMAL1 heterodimeres begin to be active again.

CLOCK/BMAL1 heterodimers also play role in the second feedback loop. Not only they enhance transcription of *Cry* and *Per* genes, but they also bind to E-boxes promoting production of REV-ERBα and REV-ERBβ repressors. These repressors are members of Orphan nuclear receptor family [42].

REV-ERBα/β repressors bind to RORE (Retinoic Acid-Related Orphan Receptor Response Element) sequence in promoter areas of *Clock* and *Bmal1* genes. After binding to this sequence and recruiting co-repressors NCoR and HDAC3 with histone deacetylase activity, this newly formed complex represses *Clock* and *Bmal1* genes' transcription.

Apart the REV-ERBα/β repressors, the RORE sequence binds also ROR transcriptional activators (RAR-related orphan receptors). The repressors and activators compete so that when levels of REV-ERBα/β repressors are low due to decreasing amount of

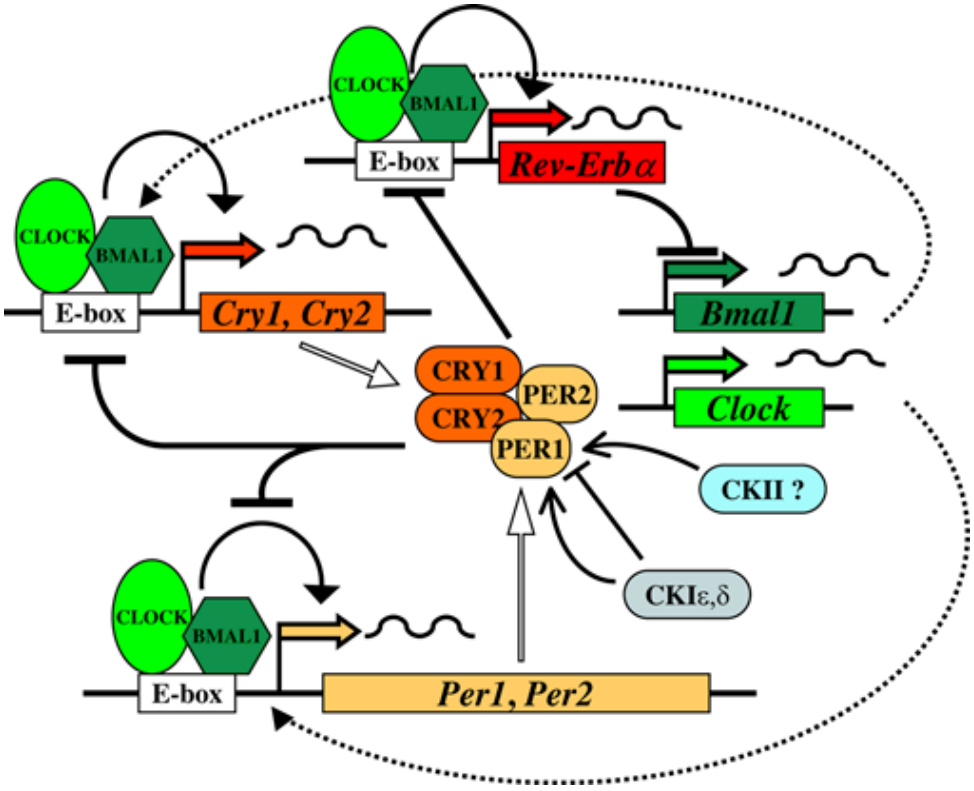


**Figure 1: Simplified scheme of mammalian molecular oscillators** When translated, polypeptides CLOCK and BMAL1 create heterodimeres and act as positive transcriptional factors. By binding to E-box they activate transcription of PER and CRY peptides. PER and CRY form heterodimeres and after phosphorylation by CKδ/ε PER/CRY complex is transported to nucleus, where after reaching a threshold it creates negative feedback and inhibits CLOCK/BMAL1 complexes. CLOCK/BMAL1 also promotes production of REV-ERBα and REV-ERBβ repressors, that inhibit transcription of *Clock* and *Bmal1* genes.(Gachon, F. et al., 2004)

CLOCK/BMAL1 complexes, ROR factors bind to RORE sequence and along with additional co-activators they enhance CLOCKs and BMAL1s production. [28,32,41]

The cycle is completed in ~24 hours and is what is called the circadian clock (Fig. 1). The period length of one's full cycle of molecular oscillations measured in environment free of any external stimuli (which means for example in constant darkness) designated as τ. Under such conditions, the clock is "free-running", i.e., not affected by external cues.

## 3. Clock synchronization

Circadian molecular oscillations described in previous chapter take place in every living cell of our body. To ensure the proper functioning of all parts of the organism, it is necessary that all constituent clocks oscillate in the tissue-specific phase. This way, all cells can align their actions to the proper time of the day.

In conditions of changing ambient environment, the circadian clock needs to retain certain extent of plasticity so it can effectively respond to those changes, for example to seasonal time changes in the day-night transitions. This change is important for example for nocturnal animals, whose activity is linked solely to the night time.

It has been shown, that various stimuli, for example light pulses, applied to a subject with specific intensity and at specific time of day have ability to either phase-advance or phase-delay the circadian cycle. This relationship between time of stimuli application and the response of the clock cycle is expressed in so called phase response curve (PRC) The shape of the PRC is stimulus-dependent; Fig.2 presents the PCR for light pulses [12,13].

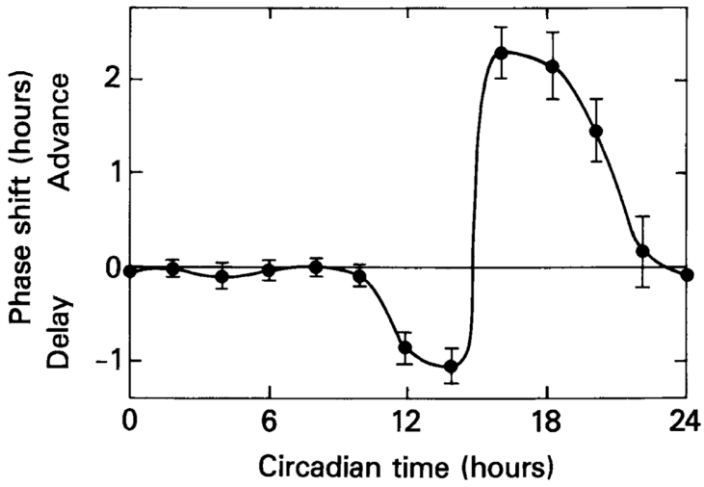


**Figure 2: Phase response curve to light** Example of effect of light pulses on circadian rhythm. Subjects were rodents kept in permanent darkness. The light impulses during specific time shifted circadian rhythm - either delayed it (impulses applied during early night) or advanced it (late night). (Lowrey, P.L. & Takahashi, J.S., 2004)

# 4. Synchronization to external stimuli

The circadian system is influenced by many external cues, generally called the *zeitgeber*s (German for "time givers"). A *zeitgeber* is an environmental factor that entrains a biological rhythm, which means that it adjusts the phase and period of the free-running rhythm (τ) according to its own period (T).

## 4.1 SCN

In mammals external cues are being processed and integrated in the internal master pacemaker and the centre of circadian rhythmicity in mammalian body located in the suprachiasmatic nucleus (SCN).

The importance of SCN as an internal master clock was proven by transplantation experiments. These experiments were performed on hamsters with τ mutation (mutation affecting period of free-running cycle) causing its shortening to 22 hours in heterozygotes and 20 hours in homozygotes. After the removal of the SCN and the confirmation of complete loss of the locomotor circadian rhythm in the recipient, the donor's SCN was implanted. Not only the circadian rhythm was re-established, but length of the circadian period matched that of the donor's genotype independently of the recipient's genotype [23].

The SCN is a part of hypothalamus and is located, as its name suggests, superior to optic chiasm, i.e. the crossing of optic nerves. It consists of two units of circa 10 000 neurons each [23]. Neurons in SCN are phenotypically divergent and they can be divided at least in two clusters - the SCN shell neurons and SCN core neurons. The main difference between them is in peptide production, ability to receive neural inputs from retina or presence of autonomous molecular oscillating mechanism.

The core SCN lies close to optic chiasm and mainly consists of neurons producing vasoactive intestinal polypeptide (VIP) or gastrin-releasing peptide (GRP). SCN core neurons directly receive neural impulses evoked by light stimuli. Neuropeptides VIP and GRP, alongside with neurotransmitters GABA (γ-Aminobutyric acid) and neuromodulator SP (substance P), which are also synthesized by the core neurons, play important role in synchronization of the SCN neurons to light.

Shell neurons surround the core neurons and unlike them, they are do not recieve direct retinal innervation. Neurons forming the SCN shell are primarily arginine vasopressin (AVP)-containing cells. These cells possess the molecular oscillation mechanism (described earlier in

chapter 2. Molecular oscillations) which is entrained by stimuli derived from neurons in the SCN core [44,45].

As already mentioned before, the SCN is not the ultimate master clock governing all circadian rhythms. The peripheral tissues and their single cells are capable of maintaining rhythmicity in circadian gene expression even in absence of the SCN. The SCN lesion resulted in gradual desynchronization among various tissue samples and their individual cellular oscillators, however their rhythmicity persisted for several days [27]. From this we can infer, that in the periphery, the SCN rather than generates the circadian rhythmicity, it synchronizes oscillators in the peripheral tissues and thus entrains their intrinsic clocks within the entire organism (Fig.3).

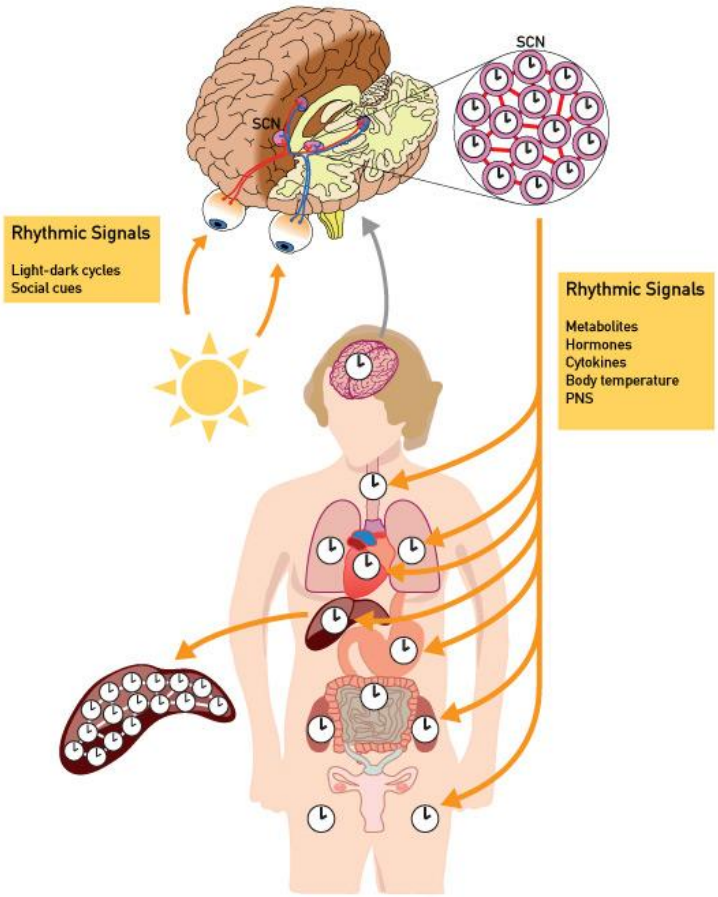


**Figure 3: Synchronizing role of SCN** Schematic image describing importance of suprachiasmatic nucleus on entrainment of internal clock. Peripheral tissues, even single cells themselves, are capable of maintaining periodicity of circadian gene expression regardless of SCN. But without SCN, their periods get diverged. Role of SCN is to synchronize these peripheral clock and to adjust them to geophysical time of day perceived by retinal photoreceptors. Synchronization is maintained by various pathways. (Bollinger, T. & Schibler, U., 2014.)

## 4.2 Synchronization to light cues

One of the most important time-setting cues for the circadian system is light (be it from natural or artificial source such as light bulbs [18]). The light-sensitivity depends on light intensity and its spectre [4,5,6].

Light stimulus is the major cue entraining the molecular oscillations within the SCN. In mammals the light is perceived solely through photoreceptors in retina and then this input is transmitted to the master circadian clock in the suprachiasmatic nucleus (SCN) [7]. The retinal cells themselves are equipped with an autonomous clock mechanism as peripheral clocks [8].

Light perception is maintained by retinal photoreceptors. The entrainment of the SCN clock remains preserved even in absence of rods and cones and had also been proven to be maintained by "blind" mammals with no visual perception as mole-rat [9,10]. Nevertheless the ability to entrain to light stimulus was lost after eye-bulb removal [11]. This finding led to extensive search for the “circadian” photoreceptors and provided evidence that subgroup of intrinsically photosensitive retinal ganglion cells (ipRGC) are the photoreceptors responsible for entrainment by light. These cells are the origin of the retinohypothalamic tract (RHT) through which the light information is transmitted to SCN. Although melanopsin is considered to be the main photopigment used in these retinal ganglion cells, existence of other photopigment is considered [24,25].

Photic cues are then processed in SCN. Neural excitation and activation of NMDA receptors by neurotransmitter glutamate causes influx of $Ca^{2+}$ ions into postsynaptic neurones. Increasing level of $Ca^{2+}$ ions causes activation of series of mitogen-activated protein kinases (MAPK), eventually leading to phosphorylation of cAMP-response-element-binding protein (CREB).

CREB affects immediate early genes, i.e. genes that are first to react to sensory stimulus. Primary genes responding to neuronal input from ipRGC are *Per1* and *Per2* genes. CREB is transcriptional factor which after phosphorylation enhances their transcription, not by binding to E-box, like CLOCK/BMAL1 complexes do, but via binding to cAMP-responsive element (CRE) in promoter region leading remodelling the chromatin [30]. Activated core neurons then send signal to shell neurons using various neurotransmitters, namely VIP, GPR, GABA and SP peptides [45]. This leads to increase in levels of PER proteins that also stabilise CRY proteins which results in modulation of the negative feedback loop [33]. This process leads to

resetting the clock phase and adjusting the period of the molecular oscillations according to solar day.

The effect of light on *Per1* and *Per2* gene expression is dependent on the time of day when the light is present. Light during the subjective day has no effect whereas light during the subjective night is effective [30].

Additionally, light is conveyed to the SCN via an alternative pathway by geniculohypothalamic tract (GHT). Unlike the RHT, which transmits the photic information to to SCN directly from photoreceptive cells in retina, this pathway is indirect. Information travels from retina through nerves to intergeniculate leaflet (IGL), and then via GHT it reaches SCN. IGL is a part of lateral geniculate complex located in rodent thalamus and is homologous with pregeniculate nucleus of primates [73].

The neurotransmitter of this pathway is neuropeptide Y (NPY). Its synthesis shows a circadian rhythm and peaks in the time of transition between light and dark. Release of this neurotransmitter is mainly regulated by photoperiod, because its circadian rhytmicity was abolished in permanently dark environment [80]. NPY is produced in the IGL and released in SCN after the stimulation of IGL caused by photic input. Inside the SCN, neuropeptide Y inhibits glutamate excitatory signalling [74], antagonist to those caused by RHT-excitation via inhibition of expression of *Per1* and *Per 2* genes [79].

In previous chapter a principle of the PRC was mentioned. It demonstrates a relationship between time of exposure to stimulus and its effect on circadian rhythm. Exposure to light at dusk phase-delays the circadian cycle whereas at dawn it phase-advances it (Fig.2). GHT is a pathway, which processes and integrates these light pulses and in the result shifts phase of circadian rhythm accordingly.

To summarize this chapter, the RHT pathway is necessary for synchronization to light-dark cycles themselves and disruption of this tract leads to total abolishment of light entrainment. The GHT co-participates in adjusting the circadian cycle to shifted environmental light and dark transitions. Lesions of IGL do not abolish entrainment to light [74].

## 4.3 Non-photic synchronization of SCN

Nevertheless the light isn't the only *zeitgeber*, that can entrain the endogenous clock. Non-photic stimuli can also entrain and adjust the circadian oscillations so that the circadian

rhythm can be entrained even in animals, which are being kept permanently in dark environment.

Non-photic inputs, just as the photic input, are being integrated in SCN, but the transmission is maintained by different pathways, depending on the input's nature. One way is via the GHT, similarly to photic input transmissions, when *zeitgeber* is transferred through IGL, and the second is through the Raphe nuclei, a serotonin-releasing part of midbrain (Fig. 4) [77].

Non-photic time-setting cues are mostly of behavioural nature including feeding-related behaviour, social interactions, arousal and exercise. The non-photic stimuli activate GHT-NPY release, which dampens *Per 1* and *Per 2* expression in the SCN neurons. The NPY levels can be increased by non-photic stimulus, e.g. exercise, in midday, when its

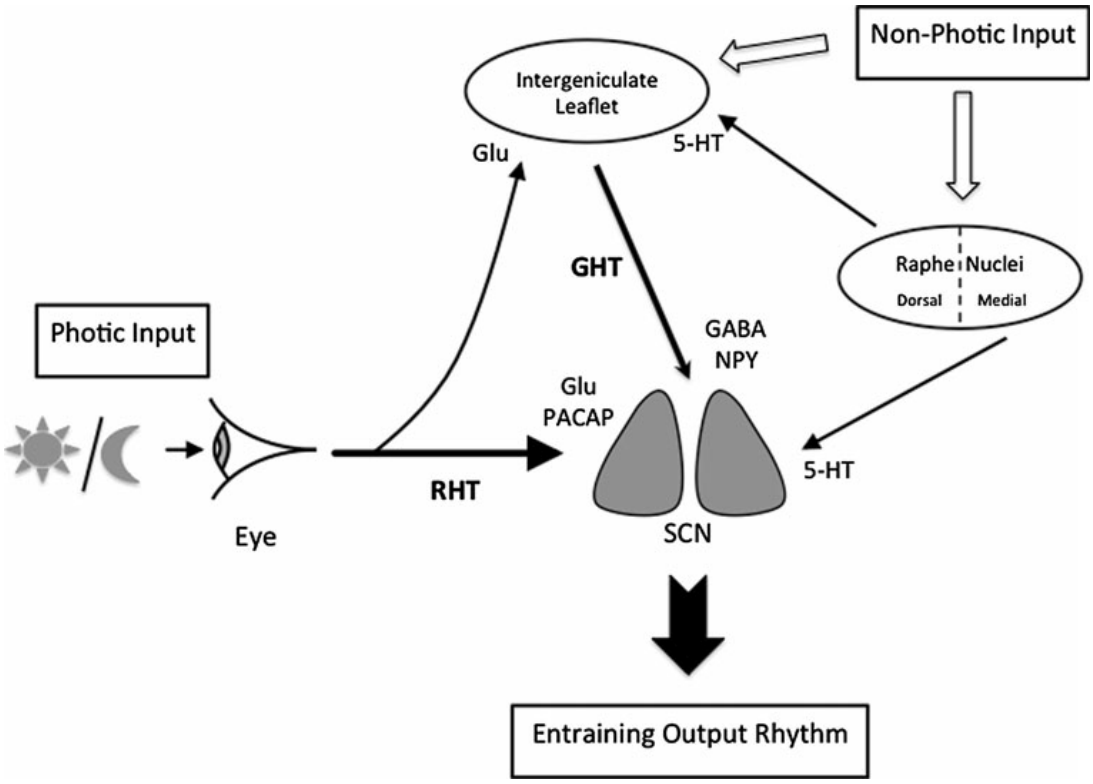


**Figure 4: Integration of external photic and non-photic inputs** External inputs are being integrated in form of generated circadian rhythm in SCN. Various neurotransmitters are being used. Photic information travels from retina to SCN either directly through Retinohypothalamic tract (RHT), using glutamate and pituitary adenylyl cyclaseactivating peptide (PACAP), or indirectly through Intergeniculate Leaflet (IGL) and Geniculohypothalamic tract (GHT) using GABA and neuropeptide Y (NPY). Non-photic information is being transmitted through IGL via the GHT and through the Raphe nuclei, using serotonin (5-HT). (Lall, G.S. et al., 2012)

concentration is the lowest [78].

The exercise-promoted NPY release is mediated by serotonin release. Serotonin, or 5–hydroxytryptamine (5-HT), is a neurotransmitter often associated with the feeling of happiness. Its release is induced by physical exercise [81] and has the ability of shifting the clock phase, application during day advances it and and phase-delays it when applied during night [77]. Serotonin-producing cells are located in Raphe nuclei, which are neurally connected with the SCN and IGL. Serotonin upon its release in SCN and IGL induces NPY production which leads to phase-shifts [78].

Neuropeptide Y also plays important role in food intake [76]. Similarly hormone ghrelin promotes feeding-related behaviour [88] and encourages food-intake related entrainment. Hormone ghrelin is secreted by gastric oxyntic cells and application of ghrelin directly to the SCN can entrain the master clock by affecting the *Per* 2 transcription just as other non-photic stimuli [88]. However this effect was achieved only in absence of other stimuli.

Beside food intake and exercise, also social interactions among individuals and enforced social routines can greatly affect one's sleeping or feeding habits in both positive and negative ways [17, 43].

Apart from behavioural inputs, also other environmental factors, for example ambient temperature which periodically changes with Earth's rotation around its axis, can act as an *zeitgeber* in ectothermic organisms, whose body temperature is directly determined by the environment (e.g. reptiles) [15]. In some endothermic organisms, i.e. organisms whose body temperature is relatively stable and independent of ambient temperature, synchronization to environmental temperature has also been observed; e.g., in animals living in habitats with extreme temperature changes such as deserts [14] but it could also noticede in species living in various climates [19]. Not only the environmental temperature, but even the body temperature in mammals shows daily rhythmicity, being an output rhythm of the SCN, and can serve as a relatively strong *zeitgeber* for the peripheral clocks (see 5.2.3.).

# 5 Internal synchronization

## 5.1 Synchronization within neurons of SCN

The internal synchronization occurs at the level of individual clocks within neurons of SCN. This process has a very special character and is solely observable in the SCN (Fig. 3).

Only a fraction of the SCN neurons, those situated in the core, are directly innervated by retinal neurons. Those neurons are the first to be responding to light input and not long after the impulse perception, increased levels of PER 1 and vasopressin are observable [75]. The core neurons then release VIP, GPR, GABA and SP peptides that send the signal to the shell neurons [44,45].

In response to time-setting light pulse, the phase is first set in core neurons and then it slowly spreads throughout the nucleus to the rest of the neurons in SCN shell. Coupling of these gradually-phased clocks in individual neurons is *in vivo* maintained through present supporting astrocytes which are connected by gap junctions and thus create glial syncytium [106]. Their role seem to be crucial for neural synchronization, since separate neurons *in vitro* didn't synchronize at all and moreover even close neurons oscillated in opposite phases [107]. As a whole unit, SCN is capable to generate coherent output and is able to send unified information about circadian period to peripheral tissues [75].

## 5.2 Synchronization of peripheral oscillators

Once the master clock is entrained, the SCN uses many different pathways to communicate with the rest of the body and synchronizes clocks in peripheral tissues (Fig. 3). These pathways cooperate and together form a sufficient synchronization signal. This is also the reason, why the phase of cells in peripheral organs is only slightly affected by elimination of one of these pathways [46]. As indicated before, they are of different nature.

### 5.2.1 Neuronal pathways

The direct pathway of signalling from the SCN to periphery is via the nervous system. Autonomous neural system can be divided into two parts - sympathetic and parasympathetic. These two neuronal systems are contradictory in their effect on target tissue. Sympathetic neural system, using adrenaline and noradrenalin as neurotransmitters, regulates many endogenous processes related to the so-called "fight or flight" response. Antagonist to it is the parasympathetic nervous system, which, by release of acetylcholine, mainly stimulates activities related to resting, digestion or reproductive activity.

Both these pathways are used by SCN for neural signalling to the peripheral organs. The crossroad redirecting output signals from SCN is paraventricular nucleus (PVN), which is a part of the hypothalamus. Signals exit SCN through vasopressin expressing cells, which are located in SCN shell. This population of cells produces also other neurotransmitters, γ–Aminobutyric acid (GABA) and glutamate. Buijs et al. [82] in his paper speculates that

presence of other co-transmitter in signalling neurons could decide nature of targetted neural pathway (sympathetic or parasympathetic).

Signal is then transmitted from PVN either directly through autonomic nervous system to its destination in peripheral organs such as adrenal gland or liver, or it indirectly synchronizes peripheral clocks via endocrine pathway of the hypothalamic–pituitary–endocrine gland axis (discussed below) [82].

### 5.2.2 Hormonal signals

The most important molecules used in hormonal signalling related to circadian rhythm are glucocorticoids and melatonin.

Glucocorticoids (GC) are steroid hormones. They are secreted by adrenal gland and distributed throughout the body and can be detected in blood serum. Once they are delivered, they bound to glucocorticoid receptors (GR) situated in cytosol of the targeted cells. The newly formed GC/GR complex then affects cellular DNA trancription [48].

The main glucocorticoid secreted in human body is cortisol. Cortisol levels are regulated by hypothalamic–pituitary–adrenal axis (HPA). Upon receiving an appropriate signal the PVN starts to release a corticotropin-releasing factor (CRF). This factor promotes secretion of corticotropin in anterior pituitary. Corticotropin, also known as Adrenocorticotropic hormone (ACTH), stimulates the secretion of cortisol [48] specifically in adrenocortical steroidogenic cells (Fig.5) [49].

Activation of the HPA is usually an immediate response to stress situations. It affects metabolism and activity of immune system. Cortisol fosters energy-storing related processes such as carbohydrate and lipid metabolism [49] or gluconeogenesis [46]. It also increases blood pressure and thus speeds up transport of oxygen and glucose. On the other hand it inhibits energy consuming activities as the immune system and it actively inhibits production of cytokines. This is also the reason, why cortisol is one of the main substances used in anti-inflammation treatment [48].

Importantly cortisol levels express robust circadian rhythmicity even when an individual is not being exposed to stress situations. Glucocorticoid expression peaks around the onset of active period, which is in the morning in diurnal organisms (such as human) or in the early night in nocturnal animals (such as mice) [49].

The rhythm in secretion of glucocorticoids is driven by the peripheral adrenal clock and affected via multiple pathways involving systemic rhythmic signals derived from the SCN. These pathways are also independent of the HPA axis. A piece of evidence supporting this hypothesis is the fact, that GC rhythm is still present in rats with surgically removed pituitary gland (hypophysectomised) and is thus independent of ACTH or CRF [53].

The daily rhythm in GC expression is affected by light cues that are processed in the SCN. Exposure to this stimulus is not accompanied by the increase in ACTH. The SCN stimulates GC production in adrenal gland via sympathetic innervation. Importance of this signalling pathway in steroidogenesis was demonstrated, when release of glucocorticoids was suppressed by transection of sympathetic nerve, whereas plasma levels of ACTH remained unchanged [50, 51].

Neural signal then reaches glucocorticoid releasing cells. Adrenaline transmits signal by binding to $\alpha_{1A}$ adrenergic receptor. This receptor activates MAP kinase pathway that results in increase of intracellular level of $Ca^{2+}$. Clock genes, whose transcription is positively affected by adrenal signalling, are *Per1* and *Per2* genes [54]. Changes in expression of these clock genes lead to resetting of the whole clock oscillatory mechanism in the adrenal gland.

The circadian clock drives rhythmically expression of clock-controlled genes. In the adrenal gland, the clock-controlled gene is *StAR* which exhibits robust rhythmicity in expression during the day [52]. StAR (Steroidogenic acute regulatory protein) is mitochondrial hormone

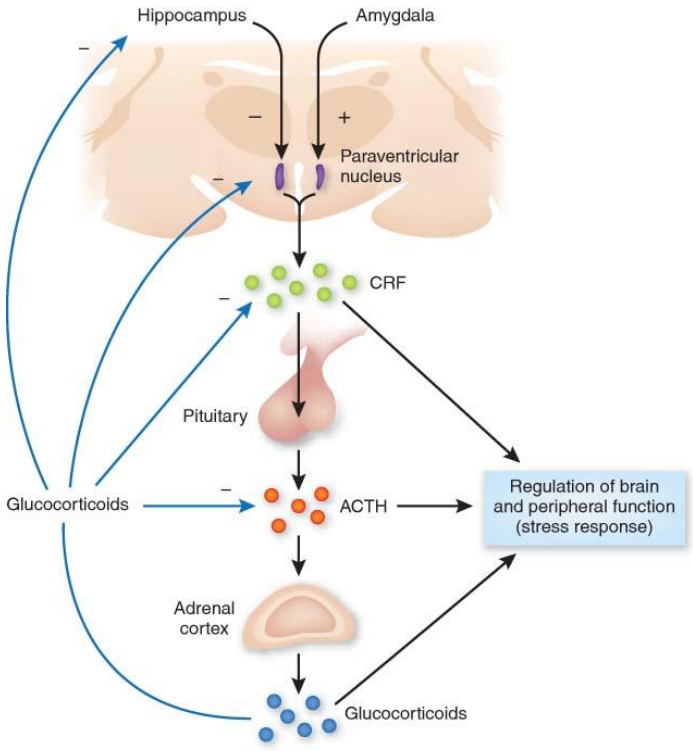


**Figure 5: The hypothalamic–pituitary–adrenal axis** Paraventricular nucleus (PVN) located in hypothalamus starts, upon receiving a appropriate signal, releasing a corticotropin-releasing factor (CRF). This factor promotes secretion of corticotropin in anterior pituitary. Corticotropin, also known as Adrenocorticotropic hormone (ACTH), stimulates the secretion of cortisol (Hyman, S.E., 2009.)

that ensures conversion of cholesterol into pregnenolone which is the first step in glucocorticoid (and specifically cortisol) biosynthesis [55]. Transcription of StAR gene is enhanced by CLOCK/BMAL1 complexes. These complexes bind to E-boxes in promoter areas. Thus, StAR is transcribed in the same phase with *Per* and *Cry* genes, that are also enhanced by CLOCK/BMAL1 heterodimeres [52].

However, the rhythm can be driven by systemic rhythmic cues because according to Son, G.H. et al. [52] both StAR and GC rhythmicity is preserved even in clock-deficient mice (BMAL1$^{-/-}$ knockdown mice), but only in presence of light cues. In constant dark, these rhythms in BMAL1$^{-/-}$ mutant mice vanished entirely.

The importance of the neural pathways in the GC rhythmic regulation is obvious from experiments previously mentioned in chapter 4 with SCN transplantations [23]; whereas the circadian locomotor activity was fully restored, the rhythmicity in endocrinal production was not present due to insufficient restoration of neural connections [56].

From both these examples we may conclude, that intact clock mechanism and neural communication between SCN and adrenal gland is essential for rhythmic production of GC.

Newly synthesized GCs are then transported in either plasma or cerebrospinal fluid

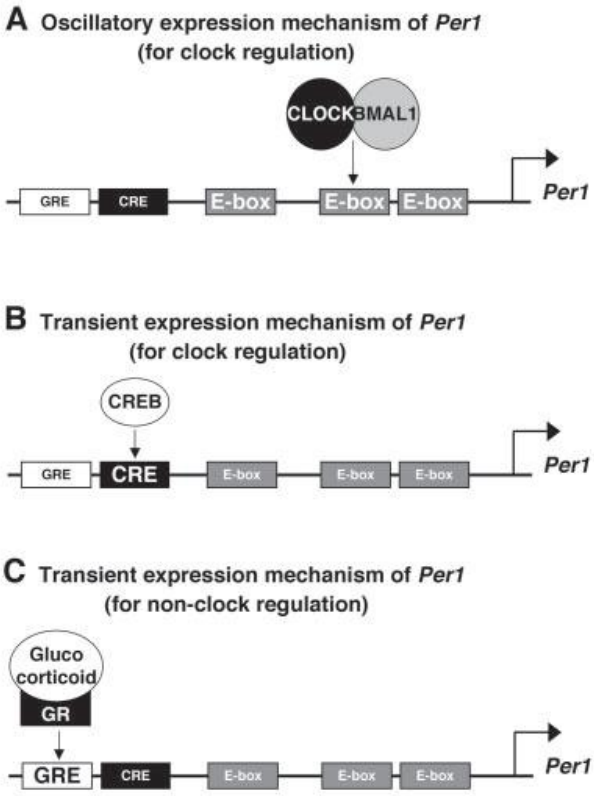


**Figure 6: Response elements within Per 1 promoter** Various response elements positively affecting transcription of *Per1* are located in *Per1* 5' upstream area. (A) CLOCK/BMAL1 heterodimers are components of basic circadian molecular clock and bind to E-box sequences. (B) Expression of Per1 is regulated via neuronal inputs by activation of MAP kinase cascade, that leads to phosphorilation of CREB, which binds to cAMP-responsive element (CRE). (C) Glucocorticoids upon forming complex with Glucocorticoid receptor (GR) bind to GRE and enhance Per1 expression. (Yamamoto, T. et al., 2005)

throughout the body. Apart from other previously mentioned functions, they can effectively entrain peripheral clocks [50]. GCs, be they natural as cortisol and corticosterone or synthesised as for example dexamethasone, affect cellular transcriptional activity by binding to glucocorticoid receptors (GR) located in cytosol. GR can be found in all peripheral tissues but not in the SCN neurons. GC are thus incapable of shifting phase of molecular oscillators of the master clock in SCN [49, 57].

After formation of GC/GR complexes, they are transported to the nucleus, where they bind to Glucocorticoid Response Element (GRE) and act as transcriptional factors. The GRE is present in promoter of *Per1* gene and its activation can enhance transcription of this clock gene (Fig.6) [58].

Glucocorticoids also enhance transcription of *Per2* gene, but the mechanism of this process seemed very unclear because no GRE sequence was found in promoter area of *Per2*. However, Cheon, S. et al. [59] demonstrated, that in fact *Per2* promoter contains GRE, but its sequence overlaps with the sequence of E-box. As described above, E-box is binding CLOCK/BMAL1 heterodimer, that also enhances *Per2* transcription. The fact that E-box and GRE sequences overlap led to an idea, that the ability of GC/GR complex to bind to GRE may be affected by the presence of CLOCK or BMAL1 proteins. Cheon, S. et al. [59] showed that transcription of *Per2* wasn't enhanced by application of dexamethasone in BMAL1$^{-/-}$ mouse embryonic fibroblasts, whereas transcription of *Per1* was. From this we may conclude, that presence of BMAL1 is necessary for glucocorticoid–mediated induction of *Per2* transcription.

Induced production of PER1 and PER2 by glucocorticoids results in phase shift of circadian phase and is indispensable for synchronization of circadian clocks in peripheral tissues to light-dark cycles [50, 52, 59].

Melatonin is probably the best known hormone associated with circadian system. It can be found in variety of organisms including plants, bacteria and vertebrates. Melatonin affects great array of physiological processes such as blood pressure, function of immune system, reproduction, oncogenesis or osteoblast differentiation and under specific conditions, it seems to have antioxidant effects. It’s role in entrainment of peripheral clocks has been often hypothesized (review [62]).

Levels of secreted melatonin show a robust circadian rhythmicity. Mammalian melatonin is produced by pineal gland and it is a derivative of amino acid tryptophan. Its production is mediated by the SCN. Apart from this, its secretion is sensitive to environmental light. Information about light is transmitted from retina to the SCN, and from there via sympathetic neural pathways to the pineal gland. The activation of $\beta_1$-adrenergic receptors by noradrenalin causes increase in production of arylalkylamine-N-acetyltransferase (AA-NAT), an enzyme involved in synthesis of melatonin. Higher AA-NAT enzymatic activity increases the production of melatonin [62, 63].

Levels of melatonin elevated in darkness and nearly undetectable during the light period (nocturnal levels of melatonin are usually 10-20 times higher than amount produced during the day [60]). As described by Kalsbeek et al. [65] it is mediated by inhibitory neurotransmitter GABA. Light inputs cause release of GABA by SCN neurons and the inhibitory signal is conveyed to the pineal gland to inhibit production of melatonin.

Duration of elevated melatonin levels reflect length of dark period, which changes annually. In animals information about the night length enables to asses the time of the year and adapt their behaviour and physiological functions accordingly [62]. The exclusive night-time production of melatonin is also present in animals independent of their diurnal or noctunal activity [61].

Melatonin is not stored in pineal cells or elsewhere and it is secreted into circulatory system immediately after its synthesis. Melatonin receptors, that bind melatonin, can be found throughout the body, i.e. in peripheral tissues and also in neurons of SCN [62]. Up to date, three subtypes of high affinity melatonin receptors have been described. Two of these subtypes, named MT-1 and MT-2, are present in mammal. They are G-protein-coupled receptors anchored in cellular membrane by seven transmembrane domains [61, 64]. MT-1 is expressed by cells of SCN, paraventricular thalamus and pituitary gland, MT-2 is as well expressed by SCN neurons and in hippocampus, human retina and brain. MT-3 receptor isn't bound with G-protein, but with quinone reductase 2, and was identified only in non mammalian vertebrates [64].

The molecular nature of melatonin effect on circadian rhythm still remains quite unclear and needs to be thoroughly examined. Nevertheless current findings suggest, that melatonin may affect expression of REV-ERB transcriptional factors, which repress transcription of CLOCK

and BMAL1, rather than *Per 1* and *Per 2* genes, clock genes whose transcription is first to be affected by e.g. light stimulus [66].

Ability of melatonin to shift the master clock in SCN and thus adjust overall circadian rhythm is well known and this feature is profusely used in medicine. Doses of melatonin are for example used in treatment of insomnia or jet-lag. Effect of melatonin on circadian system is determined by time of its administration - in the early night it advances circadian phase, in the late night it delays it. When administrated during the night-time melatonin promotes sleep in patients suffering from insomnia [60].

### 5.2.3 Body temperature

Body temperature is another *zeitgeber* that can affect rhythmic gene expression. Temperature of our body is maintained and regulated by feedback mechanism processed in preoptic anterior hypothalamus and influenced by activity of SCN [83]. The average body temperature is about 36,8˚C but it actually rhytmically fluctuates during the day. It is a result of interaction between the heat loss and production regulation and it is also determined by locomotor activity or food intake [68]. Due to the SCN regulation, temperature exhibits daily rhythm despite the individual is being exposed to constant conditions or artificial day-night periods greatly deviating from 24 hours, both procedures causing the free-running circadian rhythmicity [2,3].

Body temperature is generally fluctuating in the range of 36˚C-38˚C. As observed in humans, the lowest temperature is approximately in the middle of sleep period at about 4-6 a.m., and reaches its maximum in the late afternoon, therefore during the active period [35]. It has been observed, that even a slight difference in daily temperature fluctuations (such as 1˚C) can be a sufficient zeitgeber for the clock synchronization [68].

The mechanism is related with Heat shock factor 1 (HSF1). This factor is a member of HSF family and actively participates in clock mechanism. HSF is generally a transcriptional factor that responds to temperature changes and adjusts expression of heat shock proteins. Recent studies also show that HSF1 is able to affect transcription of clock gene *Per2* [69].

Examination of *Per2* sequence revealed, that there are two HSF1-binding sites (HSE) in *Per2* promoter. These two HSE are located 100 bp and 30 bp respectively from nearest E-box. This distance may play a rather important role, because it was shown that mutations in more proximal HSE have greater impact on *Per2* expression. This evidence suggests, that HSF1 is

actively cooperating with CLOCK/BMAL1 complex, which binds to E-box, and entrains thus *Per2* transcription [69].

Body temperature has time-setting effect only on peripheral tissues [36]. The SCN responded to heat-shock impulses only after disturbance of intercellular communication within SCN neurons [34].

### 5.2.4 Feeding

Circadian clock is not synchronized only through neural or humoral signalization. One of the most powerful *zeigebers* affecting our daily rhythm are feeding habits and associated metabolic processes.

The first idea, that circadian rhythm in peripheral tissues could be modulated by other cues than hormonal and neuronal commands from the SCN, came from the fact that samples of peripheral tissue, which after a certain period of time showed dampened oscillations in clock gene expression, became rhythmic again after exchange of culture medium, i.e. serum [38]. After series of tests it was proved, that beside many hormonal factors present in blood serum, one of key molecules initiating recurrence of circadian rhythm was glucose [37].

Addition of glucose to tissue samples affects expression of *Per1* and *Per2* genes, whose levels gradually decrease. The main candidates responsible for this occurrence were considered to be TIEG1 and VDUP1 transcriptional regulators [37]. More recent studies show that at least protein TIEG1 (transforming growth factor-beta inducible early gene 1) has a role in resetting the mammalian clock. Glucose-induced TIEG1 levels, caused a decrease in *Bmal1* levels by binding to GC boxes in *Bmal1*s promoter area. However participation of other factors in affecting transcription of *Per1* and *Per2* genes and resetting of the circadian clock is still unclear and requires further research [39].

Another factor linked to food intake, that has an effect on circadian rhythm, is cellular ratio of NADH (Nicotinamide adenine dinucleotide) and NADPH (Nicotinamide adenine dinucleotide phosphate) and their oxidized forms. This redox ratio shifts accordingly to cell's metabolism, for example the activation of metabolic processes shifts the ratio in behalf of reduced forms of these cofactors, i.e. NADH and NADPH, and during the periods of starvation the ratio is opposite. One of the sensors reacting to altered levels of oxidized and reduced cofactors is CLOCK transcriptional factor. The work of Rutter et al. [84] showed, that increased

concentration of reduced forms (NADH, NADPH) induces DNA-binding of CLOCK/BMAL1 heterodimers and higher concentration of oxidized forms ($NAD^+$, $NADP^+$) inhibits it.

Other sensor reacting to redox ratio belongs to sirtuin family. Sirtuin1 (SIRT1), also known as $NAD^+$-dependent deacetylase, is a human homologue of yeast Sir2. This enzyme helps cell to deal with oxidative stresses and affects metabolism and related gene transcription by deacetylation of histones or by modification of transcriptional regulatory proteins. It also participates in clock mechanism. Activated SIRT1 forms a complex with CLOCK/BMAL1 and interacts with both of these factors. This interaction inhibits their transcriptional activity and thus dampens *Per1* expression [100].

Similar effect on metabolic entrainment has cellular AMP:ATP ratio or more precisely activity of AMP-activated protein kinase (AMPK). AMPK is activated by elevated levels of AMP, caused for example by glycolysis, and it is also an important cellular mediator of other metabolic-related processes. Activated AMPK posttranslationally modifies, namely phosphorylates, component of clock mechanism, cryptochrome 1 (CRY 1) and decreases its stability, which consequently leads to decreased inhibition of CLOCK/BMAL1 complexes [85].

The metabolic state of cell, i.e. energy, glucose and lipid metabolism is reflected in regulated gene expression. It is carried out via nuclear receptors and about half of them is being expressed in circadian fashion [86]. Nuclear receptors, such as REV-ERBs or RORs, are direct components of the clock mechanism. Other receptors such as for example Peroxisome proliferator-activated receptors (PPARs), are also involved. Isoforms of PPAR, especially PPARα and PPARγ, interact with clock molecule PER2. PER2 interacts with N–terminal domain of PPAR, inhibits its binding to target promoters and thus affects adipogenesis, insulin sensitivity or process of fatty acids oxidation, which is controlled by these receptors [87].

Restricted feeding and controlled intake of nutrients has great impact on many organs and their manifestations. Changes in feeding habits altered gene expression in many tissues with different cell types, for example kidney, lungs, heart, pancreas or brain. But the greatest effect of restricted feeding on clock gene expression can be observed in tissues actively participating in food digestion, such as liver and other organs [40].

Liver plays a major role in metabolism of carbohydrates, proteins and lipids, and in food processing in general. From that we can conclude that food intake has great effect on oscillations of clock gene expression in hepatocytes.

Accumulated data have shown that inversion of feeding habits (e.g. daytime feeding in nocturnal mice) leads to inversion of clock gene expression in the peripheral organs. These clock gene oscillations then affect and synchronize series of food-processing related processes, such as regulation of glycogen (both glycogenesis and glycogenolysis), expression of cholesterol 7α-hydroxylase, an enzyme responsible for synthesis of bile acid from cholesterol, or even protein and amino acid metabolism. For example production of glycogen phosphorylase, a key enzyme in glycogenolysis, is induced in periods after food absorption, whereas storage of glucose in form of glycogen is promoted during food intake [40].

Liver cells adapt efficiently to these changes, but surprisingly the SCNs central clock remains completely unaffected by changes in feeding habits. That means, that hepatic clock is able to uncouple from the SCN and become the dominant entraining signal over the SCN signalling [16, 40].

Food intake also affects circadian rhythm of hormone secretion, including the above mentioned glucocorticoids, and also ghrelin, leptin, NPY and peptide YY [86].

As described before, glucocorticoids are mainly secreted during the fasting periods as part of the stress response. Deregulation of glucocorticoid rhythm leads to gradual uncoupling of other peripheral clocks from master clock in SCN, which causes, that organism is put under even more stress. This increases expression of glucocorticoids that is usually associated with chronic stress, and can result for example in obesity or insulin resistance. [67]

## 6. Circadian clock and health

From the previous chapters it is obvious that the circadian system and its components affect a great variety of biological and behavioural processes by organizing rhythmical production of many molecules throughout the human body. Whole clock mechanism is thus intertwined with metabolism, immune system, renal or cardiac function and many others. Malfunction and its desynchronization can escalate in health problems that can have direct impact on quality of life or even the lifespan.

## 6.1 External causes of clock disruption

Probably the most common way of disruption of the internal clock is by exposure to external photic cues during the night time associated with irregular lifestyle. A variety of circadian disorders falls into this category. In this case the endogenous clock of otherwise healthy individuals remains intact and its components function and communicate normally but are not properly entrained with external environment. Medical problems arise as the consequence of the clock inability to synchronize to unusual ambient time-giving cues. The clock disruption by external cues is not very harmful when occurs occasionally, but serious disease may develop when it becomes chronic.

Jet-lag, or circadian dysrhythmia, is caused by long-distance travelling. It usually occurs, when distance between the location of arrival and departure is greater than three time-zones. The severity and duration of jet-lag depends on more than one factor. Primarily, jet-lag lasts longer the more time-zones are being crossed. There is also evidence that manifestation of jet-lag is worse when travelling east than travelling west, and can worsen with age of the traveller [70].

Health problems associated with jet-lag are generally sleep disturbance, fatigue, nausea and dampened cognitive functions. The reason behind these is desynchronization of individual clocks within the circadian system. When travelling to a different time-zone, the clocks need to adapt to shifted light-dark conditions. However, different circadian rhythms adjust with different speed. For example clock tied with metabolism and food intake adjusts to changes in environment faster, than sleep behaviour. This situation causes desynchrony of clocks within different tissues throughout the body. Jet lag symptoms can be treated by melatonin which may speed up adjustment of the SCN clock to the new environment [71].

Another example of externally disrupted circadian system is via irregular working schedules called shift working. Irregular daily routine and disturbance of natural light-dark cycle can cause a variety of health problems, especially in people working on shifted shedules for long period (decades). Long-lasting impairment of circadian rhythm is being directly associated with metabolic and cardiovascular diseases, obesity and greater risk of cancer development [28, 71].

## 6.2 Disruption of internal synchronization

Disruption of circadian clock is caused not only by improper external stimulation, but also by defect in clock mechanism. Problems associated with internal clock desynchronization might also develop as one of symptoms of complex disorders.

One of examples is seasonal affecting disorder (SAD) which is in a way caused by both external and internal causes. This disorder usually affects people living in areas, where in winter the natural light periods are very short and light during the day is not intensive enough. On the other hand onset of this disorder is linked to genetic predisposition, namely sequence variations in clock genes (Per2, Arntl and Npas2) [101]. SAD is induced when the dim light during winter months is not intensive enough to act as a *zeitgeber* and the internal clock is not able to entrain itself to photic stimuli. This is accompanied by shift of intrinsic rhythms, for example temperature or melatonin rhythms. Overall desynchronization of clock mechanism escalates in series of health problems including altered sleep-wake cycles, depression or obesity [28, 89].

Malfunction of clock ability to be entrained can lead to development of sleep disorder. Circadian rhythm sleep disorders are a special category of sleep disorders and is caused by abnormalities in circadian cycle, usually in a length of free-running period (for review see [102]).

However congenital mutations in clock genes may impact not just sleep cycles. As described above, the clock mechanism is for example very closely related with regulation of digestion and dysfunction of clock factors can be a predisposition for development of type 2 diabetes mellitus and other metabolism-related dysfunctions [103, 104], but can be also harmful for development of musculoskeletal system [105] etc.

Neurodegenerative diseases are a large category of internal dysfunctions affecting circadian cycle. Currently, these diseases, which are usually linked with aging, are becoming a significant issue. These disorders, such as for example Alzheimer's disease, Huntington's disease or Parkinson's disease, mainly manifest by brain atrophy and degeneration of neural tissue in general. As described earlier, the circadian system is very tightly linked to nervous system. Therefore it is not surprising, that one of the co-morbidities accompanying neurodegenerative diseases is disruption of circadian rhythm and related processes.

Alzheimer's disease is probably the most well known disease, which affects brain tissue and cognitive functions and it is the most common cause of dementia. It is caused by multiple factors, involving both genetic and environmental factors. In this case, an increased aggregation of amyloid-β proteins results in plaque accumulation in brain. This plaque then promotes nerve degeneration [91].

Disruptions of circadian rhythms, such as disturbance of sleep-wake cycles, physiological rhythms or rhythmic melatonin secretion, occurs quite early in course of disease [91]. It is accompanied with a decrease of vasopressin and VIP in the SCN shell and core respectively [91, 92]. This disruption in SCN's integrity may lead to shift of circadian cycle and gradual desynchronization in various brain regions and thus impairment of the sleep-wake cycle. Sleep cycle disruption accompanied with shortening of REM (rapid eye motion) sleeping phase and sleep deprivation seem to promote amyloid-β peptide accumulation in brain (Fig.7) [93].

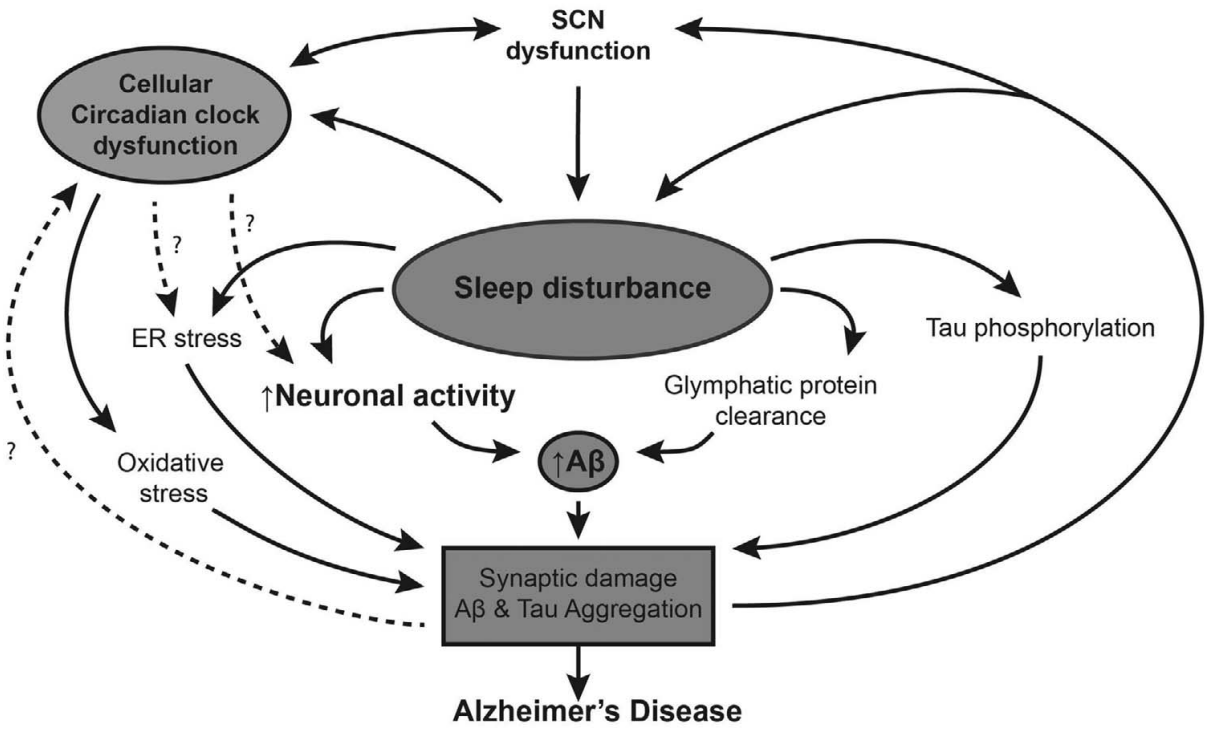


**Figure 7: Scheme of effect of Alzheimer's disease's symptoms on SCN and circadian cycle** Nerve degeneration caused by accumulation of amyloid-β plaques in SCN causes decreased number in vasopressin and VIP-expressing neurons. This disruption in SCN's integrity leads to shift of circadian cycle and gradual desynchronization in various brain regions and thus impairment of the sleep-wake cycle. Sleep cycle disruption retrogradely promotes amyloid-β peptide accumulation in brain (Musiek, E.S., Xiong, D.D. & Holtzman, D.M., 2015)

After Alzheimer's disease, the Parkinson's disease is the second most common neurodegenerative disorder. It is characterized by degeneration of dopamine-producing cells in the substantia nigra, which is a part of the midbrain. The course of Parkinson's disease is accompanied by impairment of the motor system and overall reduced motor activity.

Neurotransmitter dopamine affects plenty of physiological processes and is also involved in regulation of the circadian cycle. Beside other functions, it also plays a role in regulation of melanopsin production in retina, which shows rhythmic fluctuations depending on light and dark periods. Dopamine is also known to promote wakefulness and daytime activity [95].

Parkinson's disease negatively affects the rhythm of melanopsin production in retina alongside with other processes, which are controlled by the circadian system. Patients often suffer from disruption of activity rhythm, night-time sleep disturbance and day-time somnolence, decreased sympathetic activity and increased nocturnal blood pressure, which is most probably related with dopamine deficit [90, 95, 96].

Huntington's disease, a neurodegenerative disorder of genetic origin, also causes changes in patient's circadian behaviour. Disease is caused by autosomal dominant mutation in *Huntingtin* gene, more precisely the numerous CAG repetitions. This genetic mutation mainly affects the patient's motor, behavioural and cognitive performance, and in the most progressed stage it causes dementia. These patients also suffer from sleep disturbance and desynchronization of their intrinsic rhythms.

Disruption of circadian cycles gradually worsens with course of the disease until they are completely disintegrated [96]. It is believed, that this is caused by reduction of vasopressin and VIP-expressing neurons in SCN [99]. Neuronal degeneration is accompanied by alternations of clock oscillations, namely disruption of *Per2* and *Bmal1* gene expression in the SCN, but also in other brain areas, such as in motor cortex and striatum, and results in altogether disruption of circadian regulation within SCN [98].

Experiments performed on mice models of Huntington's disease have also revealed that changes in the SCN have impact on entrainment of the peripheral tissues. The circadian oscillations in periphery remained intact, but due to loss of entraining signals from the master clock their phases were affected, which led to overall internal desynchronization [97]. Maywood et al. [97] observed that restricted feeding resulted in synchronization of peripheral

and SCN clock oscillations and had positive impact restoration of behavioural rhythms in the mouse model of Huntington's disease.

In conclusion, nearly all studies related to neurodegenerative diseases revealed changes in the circadian rhythms and suggested that treatment of sleep-wake cycle disturbed in diseases such as Alzheimer's disease, Parkinson's disease or Huntington's disease could be an effective way to prevent or at least slow down the disease development and overall improve wellbeing of the patients.

## 7. Conclusion

Circadian rhythms are for sure very complex topic and they manifest themselves on various levels.

They can be observed in form of behaviour. In this case we are talking mostly about time-dependent feeding and rest and wake cycles. According to these cycles we may determine individuals chronotype, which is an interesting concept describing the behavioural habits determined by preferences for bed time. According to the chronotype, people can be divided in two well-known categories, i.e. so called "morning larks" and "night owls", however many do not belong into none of those two groups. Chronotype is mainly determined by length of free-running period, morning persons tend to have shorter circadian period than evening persons [21]. But belonging to a certain chronotype is determined by many other factors - behavioral habits, age or even personality [22]. This phenomenon isn't only a human's privilege and can be observed in other animals as well. Chronotype has for example a crucial role in diurnal birds reproduction - early-singing birds have greater chance of attracting females and therefore mating and producing offspring [19,20]. In other words, early bird catches the worm.

On the other hand, circadian rhythms have their base on molecular level and behavioural outcome is only one of its manifestation. Molecular clocks in form of rhythmic oscillations of so-called clock genes tick in almost every living cell in our body and entrainment of all these clocks throughput our body is crucial for right synchronization of most of the physiological processes.

Intention of this work was to describe the process of clock entrainment to external and internal cues, both photic and non-photic, and successive entrainment through neuronal

pathways, hormonal secretion (glucocorticoids, maletonin), regulation of digestion, oscillations in body temperature and many others. It is also important to understand, that proper maintenance of circadian rhythms is vital for a healthy lifestyle.

However, there are still too many things that yet remain unclear, such as concrete molecular mechanisms of clock-driven transcription of particular genes, importance of melatonin on clock entrainment or effective utilization of knowledge about circadian rhythms in modern medicine, which seem to be quite successful. Study of circadian cycles is relatively young field of study, which began to advance in course of last 30 years. During this time our understanding of processes lying behind circadian rhythms progressed greatly. Further studies might even more enlighten importance of circadian rhythms.

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