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Cirkadiánní systém během časné ontogeneze a jeho poruchy u animálních modelů a u člověka

Circadian system during early development and it´s misalignment in humans and in animal models

Disertační práce

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List of abbreviations

ADHD	attention deficit hyperactivity disorder
ANOVA	analysis of variance
AVP	arginin vasopresin
Ccg	clock controlled gene
cDNA	comlementary deoxyribonucleic acid
CK1δ	casein kinase 1 epsilon
CK1ε	casein kinase 1 delta
DBP	albumin D-site binding protein
DD	constant dark, dark/dark
DSM-IV-TR	diagnostic and statistical manual of mental disorders-IV-text revision
hnRNA	heterogeneous nuclear ribonucleic acid
LD	light/dark
LD 12:12	regime of 12 h of light and 12 h of dark
LL	constant light, light/light
mRNA	messenger ribonucleic acid
MSF	midpoint of sleep on free days
OD	optical density
RAI1	retinoic acid-induced gene 1
RF	restricted feeding regime
RT-qPCR	real-time quantitative polymerase chain reaction
SCN	suprachiasmatic nuclei
SEM	standard error of the mean
SMS	Smith-Magenis syndrome

1 ABSTRACT

The inner circadian timekeeping system rules all physiological proccesses that repeat in our body regularly every day. This system works at many different levels, from the molecular level to the level of complex behavior. Although the central clock is located in the hypothalamic brain area, the molecular mechanism responsible for the rhytmicity *per se* is present in almost every cell in the body. In humans, the misalignment of inner clock due to irregular daily schedule might lead to development of severe disorders including sleep problems, obesity, breast cancer and neurologic and psychiatric disorders. Therefore, intensive research of the circadian system is necessary for our understanding of underlying mechanisms involved in the connection between misaligned inner clock and these diseases.

During my PhD studies, we ascertained that during prenatal development in rats, fetal central circadian clock is sensitive to periodic maternal feeding. This occurs specially under conditions when the maternal circadian system is disturbed and entraining signals from the maternal central clock are lacking.

Moreover, we studied the functional state of the circadian system in children with neuropsychiatric disorders. In 10-12 year-old children with attention deficit hyperactivity disorder (ADHD), we found a shortened nighttime signal of a hormone melatonin compared with age matched control children. This might result in a shorter subjective night and consequently in a shorter sleep duration.

In children with Smith-Magenis syndrome (SMS), we proved the severely altered melatonin profiles. Moreover, we found desynchronized profiles in clock gene expression in peripheral clocks. Therefore, it is possible that the molecular clockwork of the central clock is altered in SMS children.

Finally, we ascertained that the peripheral circadian clock may sense the individual chronotype in humans even under real-life conditions.

ABSTRAKT

Vnitřní časový systém řídí všechny fyziologické procesy v našem těle, které se pravidelně opakují se zhruba denní, tj. cirkadiánní, periodou. Tento časový systém funguje na mnoha úrovních, od úrovně molekulární až po komplexní vzorce chování. Přestože jsou centrální hodiny uloženy v hypotalamu, molekulární mechanismus zajišťující tyto rytmy jako takové se vyskytuje téměř ve všech tělních buňkách. U lidí může vést narušení vnitřního časového systému vlivem nepravidelného režimu k rozvoji nejrůznějších onemocnění, např. spánkových problémů, obezity, nádorů prsou či neurologických a psychiatrických onemocnění. Proto je výzkum časového systému nezbytný pro správné pochopení mechanismů, které spojují narušený časový systém s rozvojem těchto nemocí.

V průběhu mých studií jsme objasnili, že během prenatálního vývoje potkana jsou jeho centrální hodiny citlivé na periodické krmení matky. Tento vliv se projeví především pokud je narušen časový systém matky a tím i narušeny signály vysílané z centrálních hodin matky k plodům.

Dále jsme studovali funkční stav vnitřních hodin u dětí s neuropsychiatrickými onemocněními. U desetiletých až dvanáctiletých dětí s poruchou pozornosti spojenou s hyperaktivitou (ADHD) jsme zjistili, že mají ve srovnání se stejně starými zdravými dětmi zkrácený interval vysokých nočních hladin hormonu melatoninu. To může vyústit v kratší trvání subjektivní noci a tudíž i kratší trvání spánku.

U dětí se syndromem Smith-Magenis (SMS) jsme kromě významně narušených profilů sekrece melatoninu našli i desynchronizované profily v expresi hodinových genů v periferních buňkách. Proto je pravděpodobné, že molekulární hodinový mechanismus v centrálních hodinách je u dětí se SMS narušený.

Kromě toho jsme zjistili, že lidské periferní hodiny jsou ovlivněny individuálním chronotypem, a to i pokud jsou studovány v přirozených podmínkách běžného života.

2 INTRODUCTION

2.1 Circadian rhythms

The existence of the circadian system provides organisms with an evolutional advantage of predicting changes in light, temperature and other variables that change during the day. As a consequence, the circadian system is present in almost all animals. In my dissertation thesis, I focused on the circadian system in mammals, namely in humans and rats, which is an animal model with circadian system similar to human in many aspects.

Circadian rhythms, e.g. rhythms repeating regularly with the period about 24h, occur at many levels: from molecular (transcription and translation of many genes) to physiological (rhythms in hormonal secretion or body temperature) and the whole organism (sleep/wake cycle, locomotor activity or feeding). Circadian rhythms are encoded endogenously, therefore they are not just passive reactions to rhythmically changing environment. That means that even in a nonperiodic environment, they run with the endogenous period tau. The endogenous period is species specific. In humans it differs greatly among individuals; the reported mean period length varies around 24.2 – 24.5 h, depending on the protocol used for its assessment (Carskadon et al. 1999; Czeisler et al. 1999; Kelly et al. 1999; Brown et al. 2008; Pagani et al. 2010).

At the cellular level, circadian oscillations are driven via rhythmic expression of so called clock genes and their protein products. The clock proteins control expression of their own genes as well as other genes encoding various transcription factors (reviewed in Takahashi et al. 2008) (see chapter 2.2). As an outcome of this mechanism, up to 10% of the transcriptome is under circadian control (Reddy et al. 2006). Some of these rhythmically driven genes are crucial for metabolism, cell cycle and immune response. Therefore, disruptions of the circadian regulation are associated with various diseases (reviewed in Hastings et al. 2003) (see chapter 2.4).

2.2 Molecular mechanism

At the molecular level, circadian oscillations are driven in almost every cell of the body via rhythmic expression of clock genes and their autoregulatory transcriptional-translational feedback loops. In mammals, positive elements of the primary negative-feedback loop are proteins CLOCK and BMAL1 (King et al. 1997; Hogenesch et al. 1998). Negative elements are three PER proteins (PER1, PER2, PER3) and two CRY proteins (CRY1, CRY2). During the late night, CLOCK and BMAL1 proteins accumulate in cytoplasm. They form heterodimers which translocate to nucleus and activate transcription of *Per* and *Cry* genes via binding to their E-box sequences (Gekakis et al. 1998; Bunger et al. 2000). The resulting PER and CRY proteins heterodimerize and repress the activity of CLOCK:BMAL1 complex. By this mechanism PER:CRY complex inhibit transcription of it´s own genes and therefore form a negative limb of the autoregulatory feedback loop (Shearman et al. 1997; Kume et al. 1999; Okamura et al. 1999; Vitaterna et al. 1999). During the night, the PER:CRY complex is degraded, and CLOCK:BMAL1 can therefore activate a new cycle of transcription.

In addition to the primary negative feedback loop, a secondary feedback loop which makes the clockwork more robust and precise is involved in the clockwork regulation. Genes *Rev-erbα* and *RORα* also contain E-box sequences, therefore their transcription is driven directly by the CLOCK:BMAL1 heterodimer. Their protein products compete for ROR response elements in the *Bmal1* promoter. While RORα enhances the *Bmal1* transcription, REV-ERBα represses it (see fig. 1).

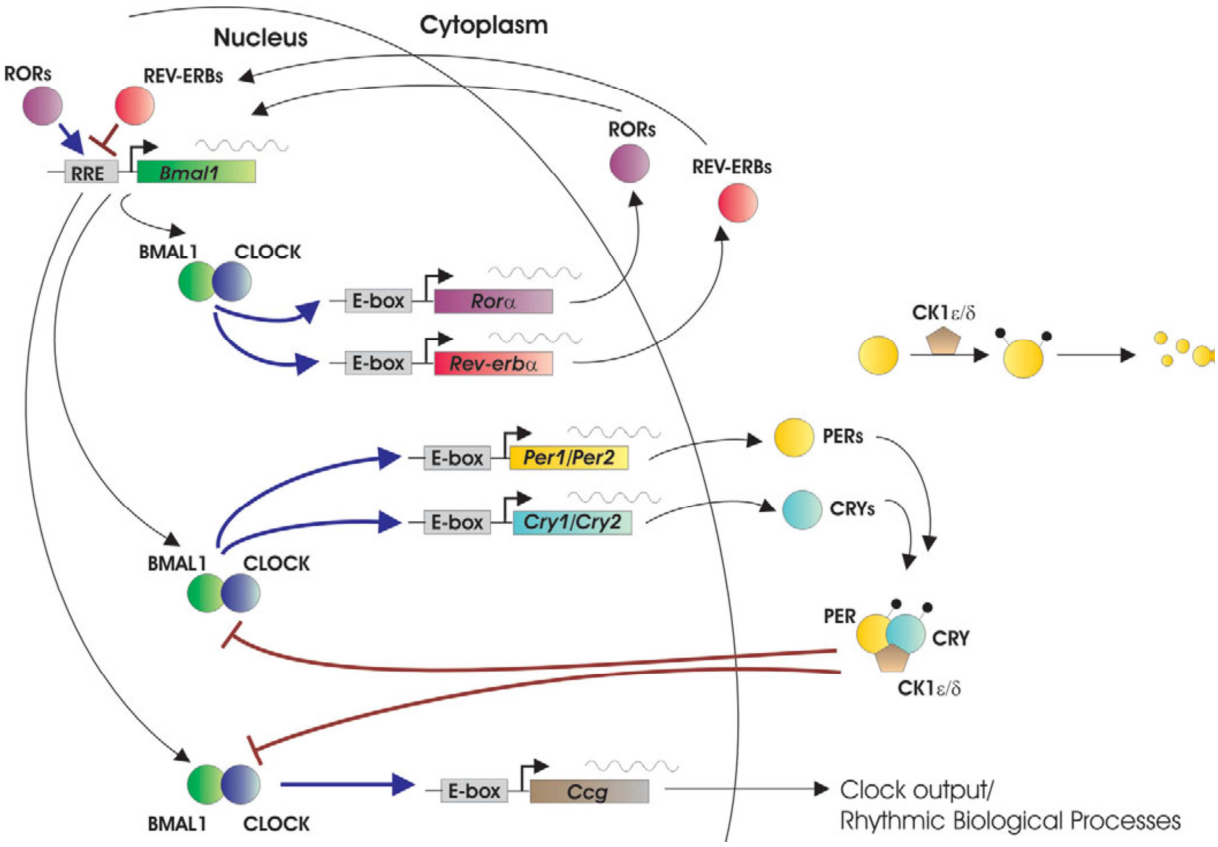
Besides the transcriptional regulation, post-translational modifications and degradation of clock proteins are crucial steps for the precisity of the circadian clockwork. A key role in post-translational modifications of clock protein stability play casein kinases *Ck1ε* and *Ck1δ*. They phosphorylate PER proteins which modifies the ability of PER:CRY complex to enter the nucleus. Moreover, phosphorylation of PER results in it´s rapid degradation, which is dependent on the ubiquitin-proteasome pathway (Lee et al. 2001; Akashi et al. 2002).

Transcription of numerous genes is regulated by the circadian clock. These genes are called clock-controlled genes (Ccgs) and are responsible for ouput rhythms because they are involved in various pathways regulating metabolism, cell cycle or immune response (rewieved in Reppert and Weaver 2001). Some of these genes are activated directly by

clock genes via their E-boxes [e.g. hormone and neurotransmitter arginin vasopresin (Avp) or a transcription factor albumin D-site binding protein (DBP)], while the others are regulated indirectly.

Figure 1: Simplified model of molecular mechanism of the circadian clockwork in mammals. (from Ko and Takahashi 2006)

Heterodimers CLOCK:BMAL1 activate transcription of Ccgs, which are responsible for rhytmic outputs and of clock genes Per, Cry, Rev-erbα and Rorα. Phosphorylated complexes of PER:CRY bind to heterodimers CLOCK:BMAL1 and inhibit transcription of their own genes. Additional feedback loop involves inhibition of Bmal1 transcription via REV-ERBα and it´s activation via RORα. For further details see chapter 2.2.



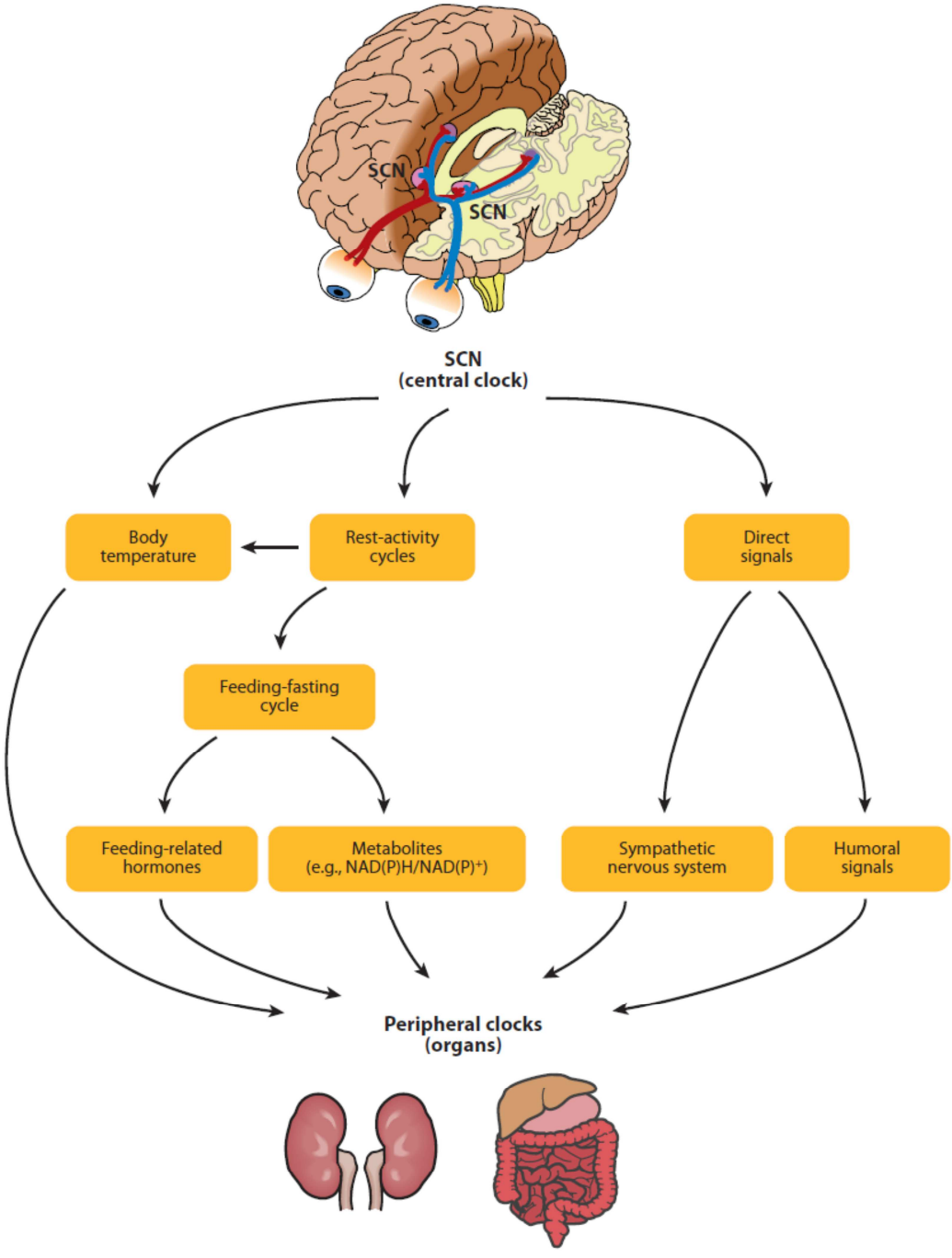
2.3 Hierarchy of the circadian system

In mammals, the central oscillator is located in the suprachiasmatic nuclei (SCN) in the hypothalamus (Ralph et al. 1990). However, almost every cell in the body contains its own oscillatory mechanism. These oscillators are referred to as peripheral clocks or oscillators. They are capable of generating self-sustaining oscillations, however, they require neuronal and humoral cues from the SCN to maintain the same phase at the organ or tissue level (Akhtar et al. 2002; Yoo et al. 2004) (see fig. 2).

Since the central oscillator is located deep in the brain, its function in humans can be evaluated only indirectly. Output rhythms, like body temperature or hormone levels, which are driven by the central clock are therefore used as markers of the functional state of the SCN. The fact that circadian molecular mechanism is present in most cells throughout the body enables also investigations of human peripheral clocks that are accessible for sampling.

<u>Fig 2:</u> Hierarchy of the circadian system (from Dibner et al. 2010)

The central clock in SCN sends direct neuronal and humoral signals to peripheral clocks. Moreover, the SCN rule the rest-activity cycle and body temperature cycle. The rest-activity cycle influences not only the body temperature but also the timing of food intake. The feeding-fasting cycle than rules turnover of metabolites and food-related hormones. All these factors act together as synchronizing cues for peripheral clocks (for more details see chapters 2.3 and 2.4).



2.4 Circadian entrainment and it´s misalignment

The circadian rhythms are entrained to the 24-h period of the solar day, namely by the light/dark (LD) cycle. The light signal is transduced directly from the retina to the central clock in the SCN (Moore 1996). The period of rhythmic processes in the SCN is therefore entrained by the solar day. This information is then sent downstream to the peripheral oscillators. When the rhythmic light signal is lacking due to exposure to constant dark (dark/dark; DD), the biological clock runs with the endogenous period tau. On the other hand, prolonged exposure to constant light (light/light; LL) disrupts the overt rhythmicity due to desynchronization among individual SCN neurons (Ohta et al. 2005).

The SCN are also entrainable by cues of a non-photic origin, though these cues are much less efficient than the LD cycle. For example, the human SCN clock may be entrained by administration of melatonin in a manner dependent on the time of delivery (reviewed in Arendt 2006). Melatonin is a hormone released from the pineal gland in a rhythmic manner. The rhythm is a direct output of the central circadian clock (see chapter 2.5 and fig. 3). The changes in melatonin levels provide the organism with information not only about the time of day but also about the season (Illnerová 1991).

In contrast to the central clock, peripheral clocks are sensitive to changes in food intake regime (reviewed in Schibler et al. 2003). Under natural conditions, the timing of locomotor activity and food intake is controlled by the SCN (see fig. 2). However, people in modern society often need to adapt their behavior to various socioeconomic demands, which forces them to be active during the night hours. Typically, this situation occurs with shift workers. When they eat at night, their peripheral oscillators might receive conflicting information about the time of the day. Signals from the SCN are dominantly entrained by the LD cycle whereas the food-processing signals are set by food intake at an improper time of day. Such a situation may cause internal desynchrony within the circadian system of the shift worker and result in aberrant temporal control of various physiological processes. The same problem may also occur in humans with temporal disruption of the sleep/wake cycle, such as those suffering from so called circadian sleep disorders like advanced sleep phase syndrome, delayed sleep phase syndrome, irregular sleep-wake rhythm, or a free-running sleep/wake cycle (reviewed in Dodson and Zee 2010), when they are forced to adapt their behavior according to the rest of the society.

Internal desynchrony of the circadian system can be induced in laboratory conditions in animal models by their exposure to restricted feeding regime (RF). It means that they have access to food only for few hours during the daytime when they are normally inactive. In adults rats, the RF entrains rhythms in clock gene expression in peripheral organs, e.g., in the liver, heart, intestine, etc., but does not affect rhythmicity in the SCN (Damiola et al. 2000; Stokkan et al. 2001; Sládek et al. 2007).

In humans, the tight relationship between the circadian system and metabolism or the cell cycle has been experimentally proven in many studies (reviwed in Savvidis and Koutsilieris 2012; Masri and Sassone-Corsi 2013). Therefore it is not surprising that misalignment of the inner clock has been associated with disorders like metabolic syndrome, obesity, type 2 diabetes, cardiovascular diseases (reviewed in Bray and Young 2007) and various types of cancer, namely the breast cancer (Megdal et al. 2005; Kubo et al. 2006). In addition, circadian abnormalities have been connected with psychiatric disorders like seasonal affective disorder, major depressive disorder, bipolar disorder (for rewiev see Lamont et al. 2010; Bunney and Potkin 2008) and neurological disorders, e.g. attention deficit hyperactivity disorder (ADHD) (LeBourgeois et al. 2004; Van der Heijden et al. 2005; **Nováková et al. 2011**) or Smith-Magenis syndrom (SMS) (Smith et al. 1998a; 1998b; Potocki et al. 2000; De Leersnyder et al. 2003; De Leersnyder 2006**; Nováková et al. 2012**).

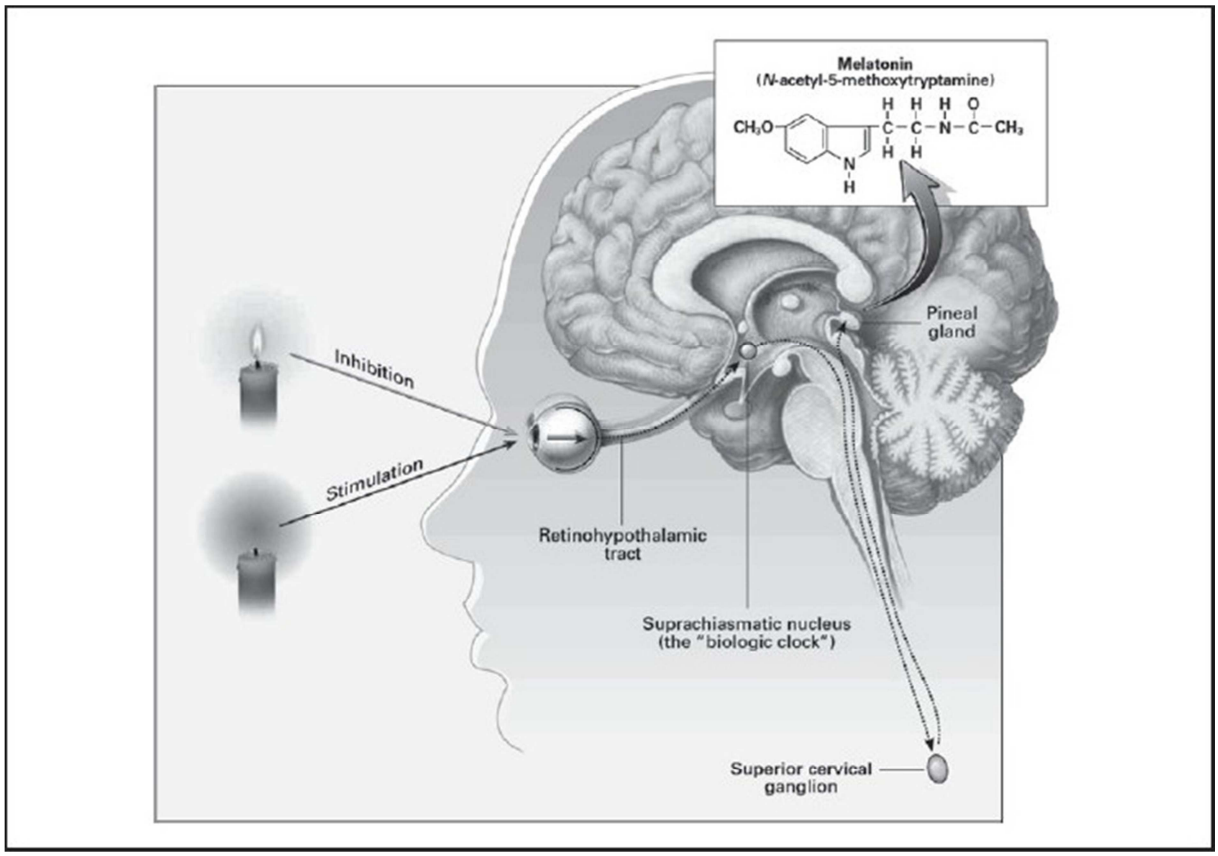
2.5 Melatonin

Melatonin is the most commonly used marker of the functional state of the circadian system, because its secretion from the pineal gland is governed directly by the SCN and its levels exhibit a robust rhythm with low levels during the day and high levels during the night (reviewed in Arendt 2006). Importantly, secretion of melatonin is resistant to external and internal cues, except for light exposure. Light suppresses melatonin levels in a dose-dependent manner (Lewy and Sack 1989; Zeitzer et al. 2000), and even low-intensity light may be effective (Bojkowski et al. 1987). However, when humans are shielded from exposure to external light, the daily profile of melatonin levels provide a precise marker of the SCN clock function. The rise and decline in melatonin levels may signal the beginning and end of the subjective night (reviewed in Arendt 2006).

Melatonin can be detected in saliva and blood, and its metabolite 6-sulfatoxymelatonin in urine. Although plasma levels of melatonin are generally 3 to 10 times higher than those found in saliva (Voultsios et al. 1997; de Almeida et al. 2011), determination of salivary melatonin is advantageous in cases when a non-invasive procedure is needed.

<u>Fig 3:</u> Light inhibition of melatonin secretion from the pineal gland (from Omar et Nabi, 2010)

Light signal from retinal photosensitive cells goes directly to the SCN via retinohypothalamic tract. Neurons from the SCN project to the pineal gland via hypothalamic paraventricular nucleus and superior cervical ganglion.



2.6 Chronotype

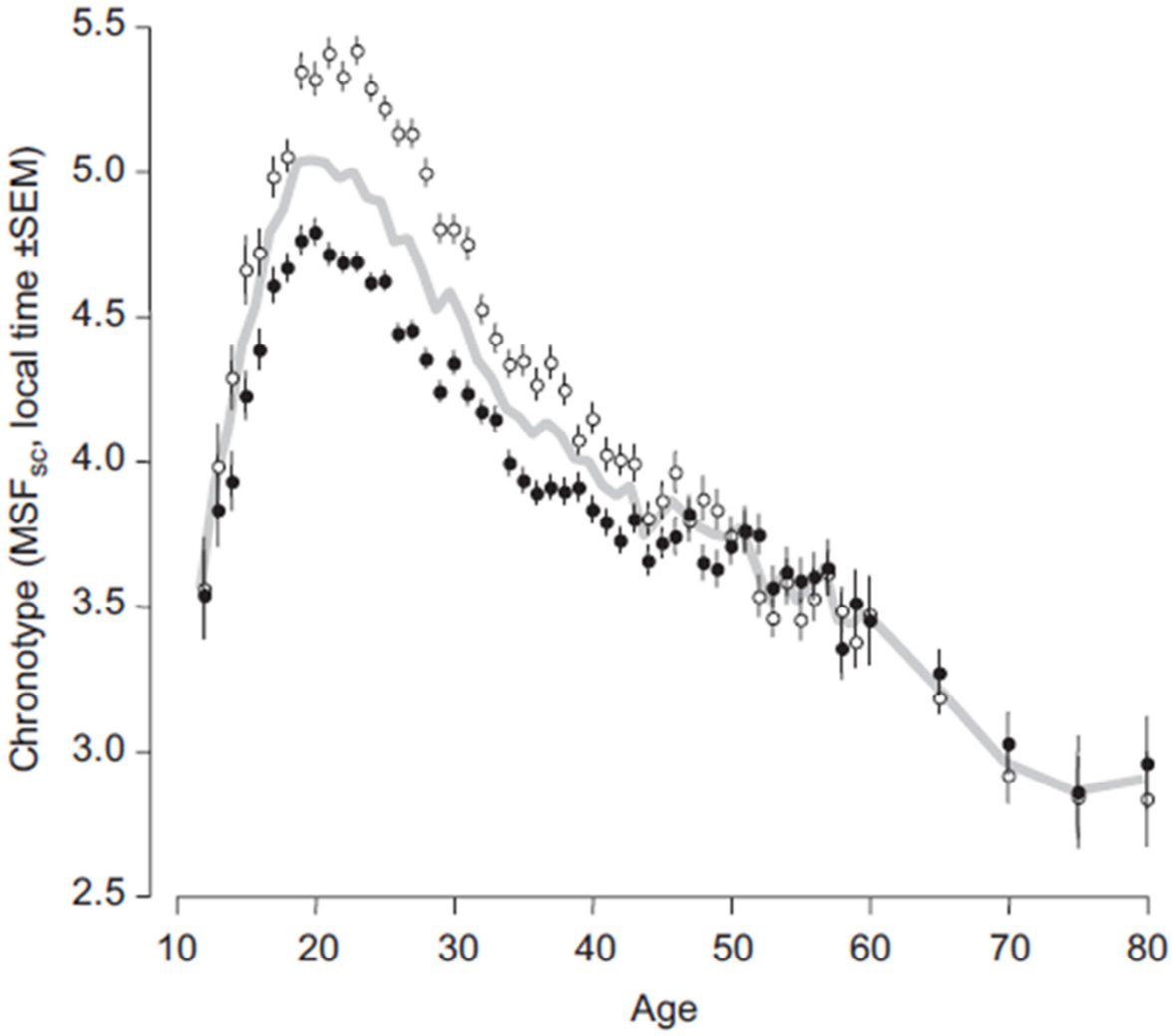
Extensive research on human circadian behavior revealed that individuals differ significantly in their specific temporal relationship to the external LD cycle (for a review see Roenneberg et al. 2007). Although exposed to similar environmental lighting conditions, some individuals prefer going to bed earlier and some later than others, which is referred to as various chronotypes. In fact, the differences are so significant that the extremely early chronotypes may wake up when the extremely late ones fall asleep. People with extremely early and extremely late chronotypes differ not only in timing of their sleep but also in timing of their melatonin and cortisol secretion and body temperature rhythms (Duffy et al. 1999; Liu et al. 2000; Bailey and Heitkemper 2001; Roemer et al. 2003).

Chronotype is influenced by environmental, social and genetic factors (Toh et al. 2001; Archer et al. 2003; 2008; Carpen et al. 2005; Vanselow et al. 2006; Barclay et al. 2010; Osland et al. 2011). The mechanisms underlying this aspect of human entrainment have not yet been fully understood. It has been suggested that chronotype is likely associated with the endogenous period length because individuals whose clock is running with longer periods tend to be later chronotypes (Duffy et al. 2001; Brown et al. 2005; Brown et al. 2008; Emens et al. 2009; Pagani et al. 2010). The intensity and duration of the actual exposure to light also plays an important role in maintaining the clock in a proper phase relationship with the external LD cycle (reviewed in Roenneberg and Merrow 2007).

Moreover, chronotype is not uniform throughout the life but changes remarkably with age (Roenneberg et al. 2004; Roenneberg et al. 2007) (see fig. 4). Chronotype also appears to be dependent on gender because in general, men tend to be later chronotypes than women (reviewed in Roenneberg and Merrow 2007). Therefore, the factors determining the individual chronotype might be very complex.

<u>Fig 4:</u> Chronotype is dependent on age and sex (from Roenneberg et al. 2007)

Black dots represent females, white dots males, grey line mean of both groups. The midpoint of sleep on free days (MSF) represents a marker of individual chronotype (Roenneberg et al. 2004).



2.7 Ontogenesis of the circadian system

2.7.1 Ontogenesis of the circadian system in rats

The largest knowledge about ontogenesis of the circadian system comes from rodent models. In rats, the prenatal period lasts about 22 days. The SCN start to form morphologically between embryonic days 14 and 18 (Ifft 1972). Only sparse synapses among the SCN neurons are detectable during the prenatal and early postnatal period. Therefore, during the prenatal period, the multilevel intercellular coupling important for complete functionality of the adult SCN clock is probably not yet present. The number of synapses gradually increases until about the postnatal day 10, when the circadian system becomes fully functional and independent of maternal signalling (Moore and Bernstein 1989).

During the embryonic development, maternal cues are responsible for setting the developing clock, while during postnatal development, the LD cycle gradually overcomes the maternal signalling (for review see Sumová et al. 2012). Prenatally, timing of the maternal behavior, which is entrained by the LD cycle (Pittendrigh 1981) may provide fetuses with information about the external time via both neuronal and humoral maternal cues (Reppert and Schwartz 1984).

The rhythmic signaling from the maternal SCN to the fetuses can be disrupted either by exposure of pregnant rats to LL or by their SCN lesion. Under these conditions, rhythms in SCN of individual pups maintain intact, however they become desynchronized within a litter (Davis and Gorski 1985; Reppert and Schwartz 1986; Shibata and Moore 1988). Therefore, maternal SCN are apparently not necessary for the development of pup´s functional clock mechanism *per se*, however, they ensure the synchronization among individual pups.

Peripheral clocks begin to exhibit rhythmicity independent of each other at various developmental stages. During the early postnatal stages, the peripheral clocks are set or driven by maternal feeding. Later, when the pup´s central clock becomes fully functional, it begins to entrain the periphery (reviewed in Sumová et al. 2012).

2.7.2 Ontogenesis of the circadian system in humans

The kowledge about ontogenesis of the circadian system in humans is still very incomplete. However, already during the end of gestation, diurnal rhythms in fetal heart rate and cortisol secretion were found (Lunshof et al. 1998, Serón-Ferré et al. 2001). In newborns, there is a progressive maturation of the circadian system outputs, with pronounced rhythms in sleep-wake cycle and melatonin secretion at about 3 months of age (reviewed in Kennaway 2000). The nighttime melatonin levels are generally low during the first 3 - 6 months, increase to a peak value at 1 - 3 years, and then slowly decline (Waldhauser and Steger 1986; Salti et al. 2000; Tordjman et al. 2005).

Human infants are able to detect light even when they are born before term (Robinson and Fielder 1990). The knowledge about the light sensitivity of infants is now being introduced to clinical praxis. Normally, neonatal intensive care units, where preterm infants are placed, use constant lighting. However, it was proven that when the preterm newborns are exposed to the regime of 12h dark and 12h light (LD 12:12), they gain weight more quickly and develop rhythms in melatonin secretion and rest-activity cycle sooner than infants under LL conditions (Kennaway et al. 1992; Rivkees et al. 2004; Watanabe et al. 2013).

Keeping the circadian system intact seems to be crucial even during the prenatal period. It was shown that disruption of the circadian system of the mothers during pregnancy by rotating shift work may result in lower weight of newborn infants (Lin et al. 2011).

2.8 Neuropsychiatric disorders associated with distruption of the circadian system

As mentioned in chapter 2.4, misaligment of the circadian system is associated not only with metabolic disorders and tumorigenesis, but also with sleep disorders, and neuropsychiatric disorders like seasonal affective disorder, major depressive disorder, bipolar disorder, ADHD or SMS. One of the aims of my thesis was to analyze in detail functional state of the circadian system in patients with ADHD and SMS.

2.8.1 Attention deficit hyperactivity disorder

ADHD is the most common childhood neurobehavioral disorder with the prevalence about 5,3 % in schoolchildren (Polanczyk and Rohde 2007). The disorder is characterized by inattention, hyperactivity, and impulsivity.

Sleep disturbances in ADHD patients, such as changes in the sleep architecture, increased daytime sleepiness, higher sleep-onset latency, and lower sleep efficiency, have been reported in some, but not all studies (LeBourgeois et al. 2004; Van der Heijden et al. 2005; Kopečková et al. 2008). Moreover, it was reported that polymorphisms in clock gene *Clock* might be associated with a greater risk of ADHD (Kissling et al. 2008). These findings suggest that a disruption of the circadian timekeeping system might be involved in the development of ADHD.

2.8.2 Smith-Magenis syndrome

SMS is a rare mental retardation syndrome (Smith et al. 1986) caused by a heterozygous interstitial deletion of chromosome 17p11.2 (Greenberg et al. 1991; Juyal et al. 1996). All patients with SMS have some degree of developmental delay and mental retardation with intelligence quotient scores ranging between 35 and 78. A short stature and brachycephaly are typical physical features (reviewed in De Leersnyder 2006).

Behaviourally, SMS patients exhibit aggressivity, self-injurious behaviour, hyperactivity with attention deficit and low sensitivity to pain. From the circadian point of view, patients suffer from severe sleep disturbances. In some studies, nearly inverted melatonin profiles with low levels during the night and high levels during the day were found (Smith et al. 1998a; 1998b; Potocki et al. 2000; De Leersnyder et al. 2001; 2003; 2006). This anomaly has been attributed to either a disrupted clock mechanism or alterations in the clock input and output pathways. The high melatonin levels during the day indicate not only aberations in circadian regulation but also the impairment of melatonin supression by light (De Leersnyder 2006).

3 AIMS OF THE THESIS

We focused on three main topics during my PhD studies.

1. **Entrainment of the fetal SCN by restricted feeding schedule of pregnant rats**

We aimed to ascertain whether exposure of pregnant rats to RF is able to entrain the circadian clock in the SCN of their fetuses during the prenatal development. We hypothesized that maternal signals from the SCN might compete with food processing signals for entraining the fetal SCN clock. To test this competition, pregnant rats were fed *ad libitum* or exposed to RF during the whole pregnancy either under an LD regime, when the rhythmic signaling from the maternal to fetal SCN was present, or under LL, when the rhythmic signaling from the maternal to fetal SCN was supposed to be abolished.

2. **Alterations of the circadian system in children with neuropsychiatric disorders**

We studied the circadian system in children with neuropsychiatric disorders, namely with ADHD and SMS.

We wanted to ascertain whether the circadian system of ADHD children differ from that of control children and how the system develops with age in both these groups of children.

In SMS patients, we aimed to find out whether the sleep and melatonin production anomalies previously reported in the literature may be due to an alteration of the molecular mechanism of the circadian clock.

3. **Variances of the circadian system in humans with extreme chronotype**

We aimed to elucidate whether the changes in the internal timing of extremely early and late chronotypes, as expressed by phases of their midpoint of sleep and melatonin secretion, can also be detected at the molecular clockwork level in subjects examined under real-life conditions. Moreover, we wanted to ascertain whether and how the phasing of the peripheral clocks correlate with melatonin profiles.

4 METHODS

4.1 Methods used in human studies

4.1.1 Participants

The ADHD subjects were recruited from the Department of Psychiatry, 1st Faculty of Medicine and General Teaching Hospital in Prague. ADHD was diagnosed by two independent child psychiatrists by means of a detailed clinical interview, which included a structured psychiatric examination, i.e., Children's Psychiatric Rating Scale (Fish, 1985), and the DSM-IV-TR diagnostic criteria for ADHD. The ADHD subjects had not been previously pharmacologically treated for ADHD and they had not exhibited any psychotropic comorbidity. At the time of the study, they had not received any medication for a general comorbidity. Only children fulfilling the criteria for the combined type of ADHD were enrolled in the study. In total, 34 children with ADHD and 43 control healthy children aged 6-12 years participated in the study.

The children with SMS were examined and diagnosed at the Pediatric Ward of the Department of Neurology, 1st Faculty of Medicine and General Teaching Hospital in Prague. In all SMS patients, genetic examination proved deletion of chromosome 17p11.2, and all exhibited the characteristic clinical features. They were free from medication for at least 2 weeks before and during the sampling period, with the exception of antiepileptic drugs in one patient. 5 children with SMS aged 3-17 years and 5 healthy, age and sex matched control children participated in the study.

The healthy control children in both studies were free from any medication and sleep disturbances, as assessed by questioning their parents.

In the last study, 95 healthy adult subjects were chronotyped with use of a modified Munich ChronoType Questionaire (Roenneberg et al. 2003). From these volunteers, 6 with the earliest and 6 with the latest chronotype were selected for the study. The midpoint of sleep on the free days (MSF) was used as a marker of individual chronotype (Roenneberg et al. 2004).

4.1.2 Saliva and buccal srubs collection

Studies were performed under real-life conditions, which reveal the actual state of the circadian system in subjects because they are exposed to various environmental and social factors that vary from day to day. Before the collection of samples, subjects were asked to behave normally, i.e., to keep their regular sleep/wake schedule, maintain their activity and go to work or attend the school. To avoid masking factors that might acutely affect the marker used for assessment of the circadian system, sampling was performed under semi-controlled conditions. One week prior the study, the subjects were asked to avoid switching on the lights at night. During the night of sampling, only a dim light of intensity lower than 20 lux was allowed. Drinking alcoholic and caffeinated beverages, using chewing gum and brushing teeth were prohibited during the entire day of sampling. 1 h before each sampling, no eating or drinking was allowed.

Samples of saliva and buccal mucosa were collected every 2 - 4 hours during the 24 h period. Saliva samples were collected either directly into the test tube or with cotton swabs. Immediatelly after the saliva collection, buccal mucosa samples were obtained by gently scratching of the inner cheek on both sides using a cytological brush.

Children in the ADHD study provided only samples of saliva every 2 hours during the 24h period. The protocol was approved by the Ethical and Research Committee of 1st Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic, and were in agreement with the Declaration of Helsinki.

Children in the SMS study and adults with extreme chronotypes provided both saliva and buccal srub samples every 4 hours during the 24-h period. The protocol and consent form were approved by the Ethical Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, and were in agreement with the Declaration of Helsinki.

4.1.3 Radioimmunoassay detection of melatonin levels in saliva

The saliva samples were stored at −20°C until assay. The Bühlmann Direct Saliva Melatonin Radio Immunoassay test kit (Bühlman Laboratories, Schönenbuch, Switzerland) was used for measuring melatonin in the samples by a double-antibody radioimmunoassay based on the Kennaway G 280 anti-melatonin antibody (Vaughan, 1993). Samples were assayed in duplicates unless the probands were not able to provide a sufficient volume of

saliva. The intra-assay coefficient of variation for sample concentration of 20,9 pg/ml was 5,9%. The mean interassay coefficient was 9,8% and the limit of assay detection was 0,2 pg/ml.

4.1.4 Quantification of clock gene expression by qRT PCR

Buccal mucosa samples were collected into RNAlater reagent (Sigma-Aldrich, St. Louis, USA) at room temperature and were then maintained at −20°C until assay.

The mRNA was isolated with the Dynabeads mRNA Direct Micro Kit (Invitrogen, Carlsbad, California, USA). cDNA was generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) in 10-μL reactions. The cDNA was then diluted 1:2 with RNase-free water and 2 μL was used for each LightCycler (Roche, Basel, Switzerland) PCR reaction in glass capillary tubes. The capillary tubes also contained 1× SYBR green PCR Mix (Sigma-Aldrich) and primers (1 μM) for a clock gene (*Per1, Per2, Rev-erbα*) or a housekeeping gene (*B2M, GAPDH*).

The PCR reactions were amplified in a LightCycler 2.0 (Roche) during 50 cycles of 15 s of denaturation at 94°C, 20 s of annealing at 60°C, and 10 s of elongation at 72°C. At the end of each run, a melting curve analysis was performed to ascertain the presence of a single amplicon. Standard curves were generated for each PCR run from the serially diluted cDNA of a human fibroblast cell line. The threshold cycles were quantified using LightCycler Analysis software version 3.5 (Roche) via the second derivative maximum method. The levels of expression of *Per1, Per2,* and *Rev-erbα* were normalized to the expression of each of the housekeeping gene, i.e., *GAPDH* and *B2M*, separately and the arithmetic mean of the relative expression was calculated. The identity of the PCR products was verified by sequencing.

4.2 Methods used in animal studies

4.2.1 Experimental animals

Prior to the experiment, adult female Wistar rats (BioTest s.r.o., Konarovice, Czech Republic) were fed *ad libitum* and maintained at a temperature of 23 ± 2 °C under LD12:12. Light was provided by overhead 40-W fluorescent tubes. Illumination was between 50 and 200 lux depending on the cage position in the animal room.

After the female rats were mated with males, pregnant rats were divided into 4 groups. Two groups remained under the previous LD12:12 regime, and rats were either fed *ad libitum* or subjected to RF. The other two groups were exposed to LL, so that light was not switched off in the evening, and rats were either fed *ad libitum* or subjected to RF. During the RF regime, rats had access to food only for a 6-h interval, starting 3 h and ending 9 h after the current and original lights on, respectively. All groups had unlimited access to drinking water during the whole experiment.

4.2.2 Locomotor activity monitoring of the rats

Locomotor activity of the pregnant rats was monitored as a marker of their SCN function. Rats were maintained individually in cages equipped with infrared movement detectors attached above the center of the cage top, which enabled detection of locomotor activity across the whole cage. A circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France) was used to measure activity every minute, and double-plotted actograms were generated for visualization of data. Resulting data, including calculations of the chisquare periodograms with $p < 0{,}001$, were analyzed using ClockLab toolbox (Actimetrics, Illinois).

4.2.3 *In situ* hybridization

The daily rhythms in the expression of *c-fos* mRNA and *Avp* hnRNA were measured by *in-situ* hybridization to detect the phase of the SCN clock of pups during the first postnatal day. *Avp* and *c-fos* were chosen as markers of the pup´s circadian system because these genes are known to cycle with a high amplitude whereas the clock genes oscilate only slightly during this early developmental stage (Sládek et al. 2004; Kováčiková et al. 2006).

Newborn pups were killed by rapid decapitation. The whole heads were immediately frozen on dry ice and stored at ‑80°C. They were sectioned into 5 series of 12-mm-thick slices in an alternating order throughout the whole rostrocaudal extent of the SCN. The cDNA fragments of rat *c-fos* and *Avp* genes were used as templates for *in vitro* transcription of ^{35}S-UTP labeled complementary RNA probes. The brain sections were hybridized with the probe for 20 h at 60°C. Following a posthybridization wash, the sections were dehydrated in ethanol and dried. Afterwards, the slides were exposed to

BIOMAX MR film (Kodak) for 10 to 14 days and developed using the ADEFO-MIX-S developer and ADEFOFIX fixer (ADEFO-CHEMIE Gmbh, Dietzenbach, Germany). Finally, slides were counterstained with cresyl violet to check the presence and midcaudal position of the SCN in each section.

Autoradiographs of sections were analyzed using an image analysis system (Image Pro, Olympus, New Hyde Park, NY) to detect relative optical density (OD) of the specific hybridization signal. In each animal, mRNA or hnRNA was quantified bilaterally, at the midcaudal SCN section containing the strongest hybridization signal. OD for each animal was calculated as a mean of values for the left and right SCN. Each measurement was corrected for nonspecific background by subtracting OD values from the same adjacent area in the hypothalamus. The background signal of that area served as an internal standard; it was consistently low and did not exhibit marked changes with the time of day.

4.3 Statistical analysis

The data for the 24-h profiles of melatonin and clock gene expression levels were depicted either individually or expressed as the mean ± SEM for each group. The 24-h profiles were analyzed by one-way or two-way analysis of variance (ANOVA) for time and group differences with subsequent pairwise comparisons by the Student-Newman-Keuls multiple range test. Student's t test was used to compare maximal levels between the groups, with $p < 0,05$ being required for significance.

Moreover, the 24-h profiles were fitted with single cosine curves (Nelson et al., 1979) defined by the equation $Y = \text{mesor} + [\text{amplitude}\cos(2\pi[\text{X-acrophase}]/\text{wavelength})]$ with a constant wavelength of 24 h. The least squares regression method was applied using Prism 5 software (GraphPad, La Jolla, USA). The acrophase and coefficient of determination R^2 (i.e., the goodness of fit) were calculated. The acrophases of the profiles were compared by Student's t test and $p < 0,05$ was required for significance.

For more detailed informations, please see the attached publications.

5 LIST OF PUBLICATIONS

5.1 Publications of the author discussed in the PhD thesis

1. **Entrainment of the fetal SCN by restricted feeding schedule of pregnant rats**

NOVÁKOVÁ, M, M SLÁDEK and A SUMOVÁ, 2010. Exposure of pregnant rats to restricted feeding schedule synchronizes the SCN clocks of their fetuses under constant light but not under a light-dark regime. *J Biol Rhythms*. vol. 25, pp. 350–360. IF 3,309

2. **Alterations of the circadian system in children with neuropsychiatric disorders**

NOVÁKOVÁ, M, I PACLT, R PTÁČEK, H KUŽELOVÁ, I HÁJEK and A SUMOVÁ, 2011. Salivary melatonin rhythm as a marker of the circadian system in healthy children and those with attention-deficit/hyperactivity disorder. *Chronobiol Int*. vol. 28, pp. 630–637. IF 4,025

NOVÁKOVÁ, M, S NEVŠÍMALOVÁ, I PŘÍHODOVÁ, M SLÁDEK and A SUMOVÁ, 2012. Alteration of the circadian clock in children with Smith-Magenis syndrome. *J Clin Endocrinol Metab*. vol. 97, pp. E312–318. IF 6,430

3. **Variances of the circadian system in humans with extreme chronotype**

NOVÁKOVÁ, M, M SLÁDEK and A SUMOVÁ, 2013. Human chronotype is determined in bodily cells under real-life conditions. *Chronobiol Int*. vol. 30, pp. 607–617. IF 4,350

5.2 Publications of the author that are not discussed in the PhD thesis

NOVÁKOVÁ, M, L POLIDAROVÁ, M SLÁDEK and A SUMOVÁ, 2011. Restricted feeding regime affects clock gene expression profiles in the suprachiasmatic nucleus of rats exposed to constant light. *Neuroscience*. vol. 197, pp. 65–71. IF 3,380

PARKANOVÁ, D, M NOVÁKOVÁ, S SOSNIYENKO and A SUMOVÁ, 2012. Photoperiodic modulation of the hepatic clock by the suprachiasmatic nucleus and feeding regime in mice. *Eur J Neurosci*. vol. 35, pp. 1446–1457. IF 3,753

SLÁDEK, M, L POLIDAROVÁ, M NOVÁKOVÁ, D PARKANOVÁ and A SUMOVÁ, 2012. Early chronotype and tissue-specific alterations of circadian clock function in spontaneously hypertensive rats. *PloS one*. vol. 7, p. e46951. IF 3,730

POLIDAROVÁ, L, M SLÁDEK, M NOVÁKOVÁ, D PARKANOVÁ and A SUMOVÁ, 2013. Increased sensitivity of the circadian system to temporal changes in the feeding regime of spontaneously hypertensive rats - a potential role for bmal2 in the liver. *PloS one*. vol. 8, p. e75690. IF 3,730

Review: SUMOVÁ, A, M SLÁDEK, L POLIDAROVÁ, M NOVÁKOVÁ and P HOUDEK, 2012. Circadian system from conception till adulthood. *Prog Brain Res*. vol. 199, pp. 83–103. IF 4,191

Review: NOVÁKOVÁ M and A SUMOVÁ, 2014. New methods to assess circadian clocks in humans. *Indian J Exp Biol*. in press. IF 1,195

6 RESULTS

6.1 Entrainment of the fetal SCN by restricted feeding schedule of pregnant rats

For detailed information and figures, please see the attached publication

NOVÁKOVÁ, M, M SLÁDEK and A SUMOVÁ, 2010. Exposure of pregnant rats to restricted feeding schedule synchronizes the SCN clocks of their fetuses under constant light but not under a light-dark regime. *J Biol Rhytmhs.* vol. 25, pp. 350–360. IF 3,385

First of all, we looked at the locomotor activity of pregnant rats to evaluate the functional state of their SCN. Pregnant rats maintained under LD12:12 and fed *ad libitum* were active mostly during the dark period of the LD cycle. However, exposure of pregnant rats to RF affected their circadian locomotor activity. The rats became active not only during the dark period but also during the time of food availability.

Pregnant rats fed *ad libitum* and exposed to LL during pregnancy showed disrupted pattern of their locomotor activity. Their locomotor activity free ran with a period of 25,3 ± 0,3 h during the first two weeks of pregnancy. Afterwards, they became gradually totally arrhythmic. On the other hand, pregnant rats maintained on LL and exposed to RF exhibited rhytmic pattern in locomotor activity with increased activity during the time of food availability. That means that RF was able to mimic the circadian rhythm in the locomotor activity of the rats. Due to the food presence during the day, the phase of the rhythm in locomotor activity was in antiphase compared with control rats maintained under LD and fed *ad libitum*.

As a next step, we measured expression of *c-fos* and *Avp* in the SCN of newborn pups. Under LD regime, expression of both genes in the SCN was rhytmical in pups born to mothers fed *ad libitum* as well as in pups born to mothers which were exposed to RF. Moreover, the phase of gene expression did not differ between these two groups. Therefore, our results revealed that exposure of pregnant rats maintained under LD12:12 to RF did not affect the *c-fos* or the *Avp* expression rhythms in the SCN of newborn pups.

In contrast, exposure of pregnant rats fed *ad libitum* to LL abolished the rhythms in *c-fos* and *Avp* expression in the SCN of their newborn pups. However, significant rhythms in *c-fos* and *Avp* expression were detected in the SCN of pups born to mothers maintained under LL and exposed to RF. The amplitude of the rhythms was lower than for control

group kept in LD and fed *ad libitum* and the maximum was in antiphase. Therefore, the results demonstrate that under LL, rhythm of the *c-fos* and *Avp* expression in SCN of newborn pups were entrained by exposure of their mothers to RF.

6.2 Alterations of the circadian system in children with neuropsychiatric disorders

For detailed information and figures, please see the attached publications

NOVÁKOVÁ, M, I PACLT, R PTÁČEK, H KUŽELOVÁ, I HÁJEK and A SUMOVÁ, 2011. Salivary melatonin rhythm as a marker of the circadian system in healthy children and those with attention-deficit/hyperactivity disorder. *Chronobiol Int*. vol. 28, pp. 630–637. IF 3,591

NOVÁKOVÁ, M, S NEVŠÍMALOVÁ, I PŘÍHODOVÁ, M SLÁDEK and A SUMOVÁ, 2012. Alteration of the circadian clock in children with Smith-Magenis syndrome. *J Clin Endocrinol Metab*. vol. 97, pp. E312–318. IF 6,568

6.2.1 Alterations of the circadian system in children with ADHD

First of all, we wanted to ascertain how the melatonin profiles change with age in the groups of ADHD and control children separately. Therefore, children from both groups were divided into 3 subgroups according to their age (groups 6-7, 8-9 and 10-12 year-old).

In the control group, we found a phase delay of melatonin profile in the oldest children, i.e. 10-12 year-old, compared with the youngest ones, i.e. 6-7 year-old. However, this trend did not hold true for the same age subgroups of ADHD children. Interestingly, we found that in the 10-12 year-old ADHD subjects, the evening melatonin onset occurred significantly later and the morning decline earlier than in the 6-7 year-old ADHD subjects. Consequently, the melatonin signal was shortened in the oldest group of ADHD children as compared with the youngest one.

Afterwards, we looked at differences in melatonin profiles between the groups of ADHD and control children. Comparison of maximal melatonin levels between ADHD and control children did not reveale any significant difference. The maximal melatonin levels did not differ even when the children were divided into the age subgroups. However, when we looked at the waveform of melatonion secretion profiles, we found significant

alterations in the ADHD group compared to healthy children. We found higher percentage of irregular melatonin profiles in the ADHD group (34%) compared with the control group (15%). Comparison of the waveforms of melatonin profiles between the entire groups of ADHD and control children (i.e., aged 6-12 years) revealed only nonsignificant tendency towards a later melatonin onset and an earlier offset in ADHD children as compared with controls. However, this trend reached significance in the oldest age group, i.e. in 10-12 year-old children. Therefore the duration of the nocturnal melatonin signal was shortened in 10-12 year-old ADHD children as compared with age-matched controls.

6.2.2 Alterations of the circadian system in children with SMS

As expected, melatonin profiles in all control children exhibited low levels during the daytime and high levels during the nighttime. On the other hand, the melatonin profiles of all SMS children showed different kinds of severe alterations, namely a phase reversion, phase advance of the rise and decline of melatonin levels, and a suppression or complete abolishment of the circadian rhythmicity.

Per1, Per2 and *Rev-erb-α* clock gene expression in buccal epithelial cells exhibited circadian variations in all control and also SMS patients. However, a detailed analysis of the expression profiles of individual clock genes revealed that although expression of *Per2* was in phase with those of *Per1* and *Rev-erb-α* in controls, the profiles were desynchronized in SMS patients.

6.3 Variances of the circadian system in humans with extreme chronotype

For detailed information and figures, please, see the attached publication

NOVÁKOVÁ, M, M SLÁDEK and A SUMOVÁ, 2013. Human chronotype is determined in bodily cells under real-life conditions. *Chronobiol Int*. vol. 30, pp. 607–617. IF 3,591

The significant correlation between the phase of the melatonin profile and timing of MSF confirmed the classification of the subjects according to their chronotype. The circadian phases of the *Per1, Per2* and *Rev-erbα* expression profiles in the oral mucosa were advanced in the early chronotypes compared with those in the late chronotypes. Moreover, the acrophases of *Per1, Per2* and *Rev-erbα* expression profiles correlated

significantly with the MSF of the individual subjects. The acrophases of the *Per1* expression profiles of individual subjects correlated significantly also with the phases of their melatonin profiles. For the *Per2* and *Rev-erbα* phases we found the same trend, however it did not reach significance.

Our results demonstrate that the individual chronotype in humans living in real-life conditions affects not only the phasing of the daily melatonin rhythm in saliva but also the phasing of *Per1*, *Per2* and *Rev-erbα* clock gene expression profiles in buccal mucosa cells.

7 DISCUSSION

In the animal study we focused on the entraining effect of a periodic maternal behavior and feeding on the fetal SCN. This effect was first observed in the SCN-lesioned pregnant rats by Weaver and Reppert (1989), who demonstrated that the circadian rhythm in drinking was restored in pups born to mothers exposed to RF during gestation independently of the postnatal care. Therefore, the synchronization was accomplished by RF during the prenatal period.

In our study, we hypothesised, that in pregnant rats, signals from maternal SCN (which are entrained by the LD cycle) and signals from the periodic maternal feeding compete for entraining the fetal SCN. Our results showed that the circadian clock in the fetal rat SCN is synchronized dominantly by signaling from the maternal SCN. When the maternal SCN is intact, it overpowers the signals from RF. However, under situation when maternal SCN rhythmicity is disturbed by prolonged exposure to LL, periodic maternal feeding and behavior might turn into a potent entraining cue of the fetal SCN.

Nevertheless, the amplitude of the restored rhythms was much lower than that of the rhythms found under LD conditions. Hence, the strength of RF to entrain the fetal clock seems to be much lower than the entraining strength of maternal SCN signaling.

When the SCN signaling remained intact, like in pregnant rats maintained in the LD regime, the phase and amplitude of the *c-fos* and *Avp* expression rhythms in the fetal SCN did not change due to exposure of pregnant rats to RF. This finding is not consistent with data by Ohta et al. (2008), who reported on entrainment of an *in vitro* rhythm in luciferase activity in the fetal SCN of *Per1-luc* transgenic rats by maternal RF. The authors found a 4.7-h advance of the SCN rhythm in fetuses of mothers maintained in an LD cycle and exposed to RF compared with fetuses of mothers fed *ad libitum*. There were significant methodological differences between the arrangement of our study and the study of Ohta et al. (2008) that might account for the different outcomes. First of all, in the study of Ohta et al. (2008), the food was restricted to only 4 h per day and animals were fasting on the day before sampling. In our study, the food was restricted to 6 h per day, and it was provided until the sampling. More importantly, we measured the activity of the SCN in newborn pups *in vivo*, whereas Ohta et al. (2008) detected the rhythms in *in vitro* explants taken during the last day of gestation. Because the fetal rat SCN neurons did not exhibit significant circadian *in vivo* rhythmicity in *Per1* expression in our previous studies (Sládek

et al. 2004; Kováčiková et al. 2006), we cannot exclude the possibility that the *Per1-luc* rhythmicity observed *in vitro* by Ohta et al. (2008) might be facilitated by a culturing procedure. The handling of explants may synchronize individual SCN cells in the explanted tissue, and the rhythm might thus be detected earlier than it would be under *in vivo* conditions. Therefore, *in vivo* conditions might provide more relevant information about the real state of the circadian system in the whole animal.

In a broader point of view, our results confirmed, that intact circadian system of pregnant mothers is necessary for correct development of circadian system of their newborns. Although we tested this hypothesis on rats, this might be hold valid also for humans. Therefore, these findings underline the importance of keeping regular daily schedule during pregnancy.

In the human studies, our interest remained focused on the circadian system during the early developmental stages. We aimed to ascertain the connections between circadian system disruptions and neuropsychiatric disorders in children, namely ADHD and SMS.

First of all, we focused on age-dependent changes in the melatonin profiles in groups of ADHD and control children separately. Interestingly, in both control and ADHD children in the range of 6-12 years of age, peak melatonin values did not decrease significantly with age, a finding that is in disagreement with the significant linear trend for a decreasing melatonin peak with age found by Attanasio et al. (1985) and Cavallo (1992). However, for this developmental period, study of Fideleff et al. (2006) did not confirm a significant decline of the urinary 6-sulfatoxymelatonin excretion rate with age. Moreover, another study reported that although the interindividual differences in melatonin production were huge, the levels remained constant in the same individuals observed from childhood until adolescence (Griefahn et al. 2003).

When we looked at changes of the waveform of melatonin profiles within the control group, we found a significant phase delay in melatonin profiles in 10-12 year-old children compared with 6-7 year-old ones. In older children, a later melatonin onset might correlate with later bedtime (Taylor et al. 2005; Crowley et al. 2006).

On the contrary, in the ADHD group, we found later evening melatonin rise but also earlier morning melatonin decline in 10-12 year-old children as compared with 6-7 year-old ones. This resulted in a shortening of the nocturnal melatonin signal in the oldest ADHD group.

As a next step, we compared melatonin profiles of the ADHD children with the control ones. Whereas ADHD children did not differ from control children in melatonin peaks, their 24-h melatonin profiles expressed as a ratio relative to the maximal melatonin levels showed some alterations. In the entire group of 6-12 year-old ADHD children, we found a trend towards shortened duration of the nocturnal melatonin signal. This occured due to a slight, but not significant, phase delay of the evening melatonin rise and a phase advance of the morning decline relative to the control group. This trend was significantly pronounced in the oldest group of 10-12 year-old children. The morning melatonin decline in the oldest ADHD group occured significantly earlier than in the oldest control group. This earlier time of the morning melatonin decline in the ADHD children compared with the control children was not due to a change in the morning entraining conditions, as both groups were adjusted to school classes starting at 08:00 h. Rather, the difference might be due to a shortening of the endogenous melatonin signal in ADHD children.

The nocturnal interval of high plasma melatonin levels and low body temperature may indicate the subjective biological night (Aeschbach et al. 2003; Illnerová et al., 2000). Extended periods of high plasma melatonin levels may facilitate longer sleep. Hence, the tendency towards a shortening of the melatonin signal, namely in older ADHD children, might be reflected in a tendency towards shorter sleep duration. Indeed, in ADHD children with sleep-onset insomnia not only delayed sleep-onset but also delayed evening melatonin rise was found (Van der Heijden et al. 2005).

Our results indicate that in older ADHD children the subjective biological night might be shortened. Consequently, the sleep period might be shortened as well. The sleep shortening and ensuing fatigue in ADHD children might partially contribute to symptoms of the disease, i.e. inattention, irritability and hyperactivity.

In the study with SMS children, we found the highly distorted profiles of melatonin secretion in all SMS children. However, only one of the five SMS children studied displayed a truly inverted melatonin rhythm with high levels during the daytime and low levels during the nighttime according to the literature (Potocki et al. 2000; De Leersnyder et al. 2001; 2003; 2006). The rest of the subjects had variable melatonin profiles, which were either depressed, phase shifted relative to controls, or were fluctuating throughout the day and night. Inverted melatonin rhythm was considered a very distinctive feature of SMS, even as a diagnostic marker. However, our results, which are in agreement with

recent studies of other groups (Boudreau et al. 2009; Chik et al. 2010), do not fully support this idea. Melatonin secretion profiles are more likely severely disrupted, however, not necessarily totally inverted in all SMS patients.

Anomalies in the 24-h melatonin profiles of the SMS subjects have been attributed either to an alteration of the SCN circadian clock, to distorted pineal gland function, to damped sensitivity of the melatonin production to light or to defective pathways mediating temporal information from the clock to the pineal gland (for review see De Leersnyder 2006). In humans, it is difficult to directly test the integrity of the central clock in the SCN, and, therefore, only output rhythms driven by the master clock may serve as markers of SCN function. The presence and normal phase of circadian rhythms in cortisol, growth hormone and prolactin (for review see De Leersnyder 2006), favored the possibility that the central SCN clock is not affected in SMS patients. However, rhythms in growth hormone and prolactin might also be related to changes in posture due to activity/rest cycles and the fully endogenous nature of these hormonal rhythms still needs to be proven.

To elucidate the question of whether the circadian system of SMS patients is intact or distorted, we measured expession of clock genes *Per1, Per2* and *Rev-erbα* in buccal mucosa cells. We found a desynchronization in phasing of individual clock genes in the SMS group. The expression profile of clock gene *Per2* was out of phase compared with profiles of *Per1* and *Rev-erbα*. Thereby it seems that the mucosal circadian clock is apparently running in SMS subjects but might be less synchronized with the external environment, likely due to weaker signals from the SCN.

According to our results, it appears that melatonin rhythm disruptions reported in SMS children don´t have their origin in distorted pineal gland as previously hypothesised. Since the disorganization of circadian rhythms was found also at the level of clock gene expression in peripheral cells, we hypothesise that the central oscilator in the SCN might be less well organized and less robust and as a consequence might send weaker signals to both the pineal gland and the peripheral organs.

In a recent study, Williams et al. (2012) studied the retinoic acid-induced gene 1 (*RAI1*), a transcription factor coded on a the deleted SMS region (chromosome 17p11.2). A point mutation in *RAI1* leads to phenotype similar to SMS (Slager et al. 2003; Bi et al. 2004; 2006). Therefore, *RAI1* appears to be the critical gene for SMS phenotype. Williams et al. (2012) discovered that *RAI1* is a positive transcriptional regulator of a canonic clock gene *Clock*. Haploinsufiency of *RAI1* resulted in the transcriptional desregulation of the

circadian clock in human fibroblasts and caused altered expression and regulation of multiple circadian genes, including *Per2, Per3, Cry1* and *Bmal1*. These results support the hypothesis that the molecular clockwork in SMS is altered.

Therefore it is possible that decrease or absence of circadian regulation of melatonin levels shown in SMS patients might be due to a decline in robustness of the central clock. Because the central clock is directly entrainable by light, the light therapy (besides the melatonin treatment) might become an effective treatment for some sleep problems of SMS children.

In the last study we focused on the circadian variation in healthy adult subjects with extreme chronotype.

We found out that not only the melatonin secretion acrophases, but also the acrophases of the clock gene expression profiles occurred earlier in individual subjects with the early chronotypes compared with those with the late chronotypes. This was demonstrated for all three studied genes, i.e. *Per1, Per2* and *Reverbα*. Moreover, the correlation between MSF and both melatonin and clock gene expression acrophases was proven for the groups of early and late chronotypes as well as for the individual subjects of each group.

However, when the phases of clock gene expression were correlated with the phase of the melatonin profile in each individual, a significant correlation was observed only for *Per1* expression. Therefore, whereas *Per1* expression correlated with MSF and melatonin profile, *Per2* and *Reverbα* expression only correlated with MSF but not with the timing of melatonin maximum. Theoretically, this might be caused by the fact that the sampling was provided only in 4-h intervals, which represents the main limitation of this study. However, the sampling interval couldn´t be shorter to prevent subjects' discomfort from oral mucosa damage due to the repeated brushing to sample the mucosal cells.

Nevertheless, all the data together demonstrate that chronotype is not only reflected in the phasing of the central clock in the SCN as revealed by the phase of the melatonin secretion pattern, but also in the phasing of the peripheral clocks as revealed by the expression profiles of *Per1, Per2* and *Reverbα* in buccal mucosa. Our study represents the first demonstration that the human peripheral circadian clock may sense the individual's chronotype under field study conditions.

Therefore, the results indicate that individuals with early or late chronotypes have their circadian system phased globally differently compared with major population. Nevertheless, these individuals must often adapt their behavior to the social schedules dictated by the majority. However, proper internal phasing of the circadian system that is in synchony with the external environment appears crucial for our health (for review see Takahashi et al. 2008). The situation, when the internal clock is out of phase with the outer world, which occurs when extreme chronotypes are forced to adapt to social time, is called the social jet lag (Wittmann et al. 2006). Social jet lag more likely occurs in late chronotypes, whose sleep onset is determined by their inner clock but the wake-up time is forced by social cues. Recently, accumulated data have suggested that the late chronotypes may be more susceptible to mood disorders (Kitamura et al. 2010). Moreover, chronotype influences the individual tolerance to shift work. While late types usually struggle with morning shifts, early types have troubles with night shifts (Juda et al. 2013).

Therefore, understanding the mechanisms underlying the chronotype in real life appears important for human well-being and for revealing interactions of temporal timing with various aspects of human behavior, brain functions, and physiological processes in our body.

8 CONCLUSIONS

1. Entrainment of the fetal SCN by restricted feeding schedule of pregnant rats

We proved that signals from maternal SCN are the main synchronizing cues for the fetuses. However, under situation when maternal SCN rhythmicity is disturbed by prolonged exposure to constant light, periodic maternal feeding might turn into a potent entraining cue of the fetal SCN.

2. Alterations of the circadian system in children with neuropsychiatric disorders

ADHD children did not differ from controls in the means of maximal melatonin levels. However, when the waveforms of melatonin secretion profiles were compared, the melatonin signal was shortened in ADHD children compared with controls, namely in the oldest group. Therefore, our results indicate that in 10-12 year-old ADHD children, the subjective biological night might be shorter compared with age matched controls.

In children with SMS, the disorganization of circadian rhythms was found not only in melatonin secretion profiles, but also in profiles of clock gene expression in peripheral cells. Therefore it seems that in SMS patients, the molecular clockwork of the central oscilator in the SCN is probably less well organized and less robust compared with controls.

3. Variances of the circadian system in humans with extreme chronotype

We ascertained, that chronotype is not only reflected at the level of sleep timing and melatonin secretion, but also at the level of phasing of the peripheral clocks in subjects examined under real-life conditions.

9 RESUME

In my PhD thesis, I focused on the circadian system during childhood in humans and during early developmental stages in rats. Moreover, the functional state of circadian system was studied in volunteers with extreme chronotype and with neuropsychiatric disorders, namely ADHD and SMS.

In the broader view, all the results together underline the importance of maintainance of the intact inner clock by keeping the regular daily regime. Specially during pregnancy, irregular feeding and light regime might result in abberant development of newborn´s inner clock.

Moreover, in humans with extreme chronotype, especially in the late types, the misalignment of inner clock with external environment might occur. This misalignment appears to be very complex, including not only the master clock but also the peripheral clock.

Finally, the demonstration of specific disruptions of the inner timing system in neuropsychiatric disorders might be heplfull for development of new treatments based on chronotherapy, specially the light therapy. Chronotherapy might turn out to be usefull specially for treatment of sleep irregularities.

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SUPPLEMENT: PUBLICATIONS